

**TRANSMITTAL OF  
UTILITY  
APPLICATION  
UNDER 37  
C.F.R. § 1.53**

Attorney Docket No.	22908-1227B
First named inventor	Barbas, Carlos F., III
Express mail label #	EL406143459US
Date of mailing	June 2, 2000

SEC Box

**Application Elements**

1. ☒ Fee Transmittal Form
2. ☒ Specification containing 139 pages (including claims and Abstract) and a Sequence Listing (46 pages).
  - a. Title: **LIGAND ACTIVATED  
TRANSCRIPTIONAL REGULATOR  
PROTEINS**
  - b. Number of claims: 73
3. ☒ 27 sheets of drawings with 27 Figs.
4. ☐ Unexecuted Declaration listing name of inventor
5. ☒ Sequence Listing
  - ☒ Paper copy (identical to computer copy)
  - ☒ Computer readable copy
  - ☐ Verified statement

**Accompanying Application Papers**

6. ☐ Copy of assignment from prior
7. ☐ Small Entity Statement
8. ☐ Preliminary Amendment
9. ☒ Return Receipt Postcard

3511 U.S. PTO  
09/586625

06/02/00

**SIGNATURE OF ATTORNEY/AGENT**

HELLER EHRMAN WHITE &amp; McAULIFFE LLP

*Stephanie Seidman*  
Registration Number: 33,779

☒ Benefit of priority claimed under 35 U.S.C. § 120 to U.S. application Serial No. 09/433,042, filed October 25, 1999, which is incorporated by reference in its entirety

**CORRESPONDENCE ADDRESS**

NAME	Stephanie Seidman Registration No. 33,779 Heller Ehrman White & McAuliffe LLP	
Address	4250 Executive Square, 7th Floor, La Jolla, CA 92037	
	Telephone: 858.450-8400	Facsimile: 858.587-5360

00/20/96  
3511 U.S. PTO

09586625-060200

## LIGAND ACTIVATED TRANSCRIPTIONAL REGULATOR PROTEINS

Work described herein was supported by National Institutes of Health NIH Contract No. GM53910. The United States Government has certain rights in such subject matter.

### RELATED APPLICATIONS

This application is a continuation-in-part of U.S. application Serial No. 09/433,042, filed October 25, 1999, to Carlos F. Barbas III, Michael Kadan, and Roger R. Beerli, entitled "**Recombinant Ligand Activated Transcriptional Regulator Polypeptides.**" U.S. application Serial No. 09/433,042 is herein incorporated by reference in its entirety.

### FIELD OF THE INVENTION

The field of this invention is the regulation of gene expression. In particular, ligand-activated fusion proteins (also referred to herein as chimeric regulators) and the use thereof for regulation of gene expression are provided. The fusion polypeptides contain a DNA binding domain containing one or a plurality of zinc finger polypeptide domains and a ligand binding domain (LBD) derived from an intracellular receptor.

### BACKGROUND OF THE INVENTION

Intracellular receptors are a superfamily of related proteins that mediate the nuclear effects of a variety of hormones and effector molecules, include steroid hormones, thyroid hormones and vitamins A and D. Members of this family of intracellular receptors are prototypical ligand activated transcription factors. These receptors contain two primary functional domains: a DNA binding domain (DBD) that contains about sixty-six amino acids and a ligand-binding domain (LBD) located in the carboxyl-terminal half of the receptor that has about 300 amino acids. The receptors are inactive in the absence of hormone (ligand) by virtue of association with inactivating factors, such as heat shock proteins. Upon ligand binding, the receptors dissociate from the inactivating complex and dimerize, which renders them able to bind to DNA and modulate transcription.

For example, for the steroid receptors, binding of a steroid hormone to its receptor results in receptor protein homodimerization and subsequent binding to the "steroid response element" (SRE) DNA sequence in nuclear DNA. Conformational changes in the receptor associated with ligand binding results in the recruitment of other transcriptional regulatory proteins, called co-activators, that regulate the transcription from promoters adjacent to the SRE binding sites.

Modified steroid hormone receptors have been developed for use for regulated expression of transgenes (see, *e.g.*, U.S. Patent No. 5,874,534 and published International PCT application No. WO 98/18925, which is based on U.S. provisional application Serial No. 60/029,964) by modifying the ligand specificity of the LBD. In addition, the DNA binding domain of the receptor has been replaced with a non-mammalian DNA binding domain selected from yeast GAL4 DBD, a viral DBD and an insect DBD binding domain to provide for regulated expression of a co-administered gene containing a region recognized by the non-mammalian DBD. These constructs, however, have several drawbacks. The non-mammalian DBD is potentially immunogenic and the array of sequences recognized by these DBD is limited, thereby severely restricting gene targets.

Therefore, there remains a need for more versatile gene regulators. It is an object herein to provide polypeptides that function as versatile regulators of gene expression.

#### **SUMMARY OF THE INVENTION**

Polypeptides that function as ligand activated transcriptional regulators and nucleic acid molecules encoding such polypeptides are provided. The polypeptides are fusion proteins that are ligand activated transcriptional regulator that can be targeted to any desired endogenous or exogenous gene. Variants of the fusion protein can be designed to have different selectivity and sensitivity for endogenous and exogenous ligands.

Nucleic acid molecules encoding the fusion proteins, expression vectors containing the nucleic acids and cells containing the expression vectors are provided. The fusion protein or nucleic acids, particularly vectors, that encode the fusion protein can be introduced into a cell and, when expressed in the cell, regulate gene expression in a ligand-dependent manner.

### **Fusion proteins**

The fusion proteins provided herein contain a ligand binding domain (designated herein LBD) from an intracellular receptor, preferably a LBD that has modified ligand specificity compared to the native intracellular receptor from which the LBD originates, and a nucleic acid binding domain (designated herein DBD) that can be tailored for any desired specificity. The fusion proteins may also include a transcriptional regulating domain (designated herein TRD), particularly a repressor or activator domain. The domains are operatively linked whereby the resulting fusion protein functions as a ligand-regulated targeted transcription factor.

When delivered to the nucleus of a cell, the domains, which are operatively linked, together act to modulate the expression of a targeted gene, which may be a native gene in a cell or a gene that also is delivered to a cell. Hence the targeted gene can be an endogenous cellular gene or an exogenously supplied recombinant polynucleotide construct. The fusion protein may also include a transcriptional regulating domain that is selected to activate, enhance or suppress transcription of a targeted gene.

In one embodiment, the fusion protein is constructed from components highly similar to human proteins, preferably components that are about 80% more preferably about 85%, most preferably at least about 90% identical in amino acid sequence to the corresponding human domain. In another embodiment, the fusion protein binds to a naturally occurring gene and modulates the transcription of the naturally occurring gene in a ligand-dependent way. In another embodiment, the fusion

protein binds to an exogenously supplied recombinant construct and modulates the transcription of the exogenously supplied recombinant construct in a ligand-dependent way.

In a preferred embodiment, the isolated recombinant fusion protein forms a dimer when bound to a polynucleotide. The dimer can be a homodimer or a heterodimer. In one embodiment, the dimer includes at least one DNA binding domain, at least one, preferably two, ligand binding domains and at least one transcription modulating domain. In heterodimers, the dimer can include two different DNA binding domains, two different ligand binding domains or two different transcription modulating domains. One exemplary heterodimer includes at least three zinc finger modular units, two different ligand binding sites and a transcription modulating domain.

Exemplary fusion proteins containing zinc fingers and LBD that are non-responsive to estrogen, and that are induced by synthetic non-steroidal drugs that are routinely used for clinical treatments are described; these regulators provide ligand-dependent gene activation. Exemplary fusion proteins comprise the sequence of amino acids encoded by the open reading frame set forth in each of SEQ ID Nos. 1-18.

The fusion proteins can be used in plant species as well as animals. Transgenic plants resistant to particular bacterial or viral pathogens can be produced.

#### **Ligan Binding Domain (LBD)**

The LBD is derived from an intracellular receptor, particularly a steroid hormone receptor. The receptors from which the LBD is derived include, but is not limited to, glucocorticoid receptors, mineralocorticoid receptors, thyroid hormone receptors, retinoic acid receptors, retinoid X receptors, Vitamin D receptors, COUP-TF receptors, ecdysone receptors, Nurr-1 receptors, orphan receptors and variants thereof. Receptors of these types include, but are not limited to, estrogen receptors, progesterone receptors, glucocorticoid- $\alpha$  receptors, glucocorticoid- $\beta$

receptors, androgen receptors and thyroid hormone receptors. LBDs preferably are modified to alter ligand specificity so that they preferentially bind to an exogenous ligand, such as a drug, compared to an endogenous ligand.

When intended for human gene therapy, the ligand binding domain preferably retain sufficient identity, typically at least about 90% sequence identity to a human ligand binding domain, to avoid substantial immunological response. A single amino acid change in the LBD can dramatically alter performance of the protein.

The LBD is preferably modified so that it does not bind to the endogenous ligand for the receptor from which the LBD is derived, but to a selected ligand to permit fine tuned regulation of targeted genes. Hence, in certain embodiments, the ligand-binding domain has been modified to change its ligand selectivity compared to its selective in the native receptor. Preferably the modified ligand-binding domain is not substantially activated by endogenous ligands. Any method for altering ligand specificity, including systematic sequence alteration and testing for specificity, and selection protocols (see, *e.g.*, U.S. Patent No. 5,874,534 and Wang *et al.* (1994) *Proc. Natl. Acad. Sci. U.S.A.* 91:8180-8184) can be used.

#### **Nucleic acid binding domain (DBD)**

To achieve targeted and specific transcriptional regulation the DBD includes at least one zinc finger modular unit and is engineered to bind to targeted genes. The zinc finger nucleic acid binding domain contains at least two zinc finger modules that bind to selected sequences of nucleotides. Any zinc finger or modular portions thereof can be used. The DBD replaces or supplements the naturally-occurring zinc finger domain in the receptor from which the ligand binding domain is derived.

The nucleic acid binding domain (DBD) includes at least one, preferably at least two, modular units of a zinc finger nucleic acid binding polypeptide, each modular unit specifically recognizing a three nucleotide

sequence of bases. The resulting DBD binds to a contiguous sequence of nucleotides of from 3 to about 18 nucleotides.

As noted, the DBD contains modular zinc-finger units, where each unit is specific for a trinucleotide. Modular zinc protein units can be combined so that the resulting domain specifically binds to any targeted sequence, generally DNA, such that upon binding of the fusion protein to the targeted sequence transcription of the targeted gene is modulated.

The zinc finger-nucleotide binding portion of the fusion protein can be derived or produced from a wild type zinc finger protein by truncation or expansion, or as a variant of a wild type-derived polypeptide by a process of site directed mutagenesis, or by combination of a variety of modular units or by a combination of procedures.

Cys<sub>2</sub>His<sub>2</sub> (C2H2) type zinc finger proteins are exemplary of the zinc fingers that can replace the naturally occurring DNA binding domain in an intracellular receptor, such as the C4-C4 type domain in a steroid receptor, to form a functional ligand-responsive transcription factor fusion protein. By virtue of the zinc finger, the resulting fusion protein exhibits altered DNA binding specificity compared to the unmodified intracellular receptor.

The optimal portion of the ligand binding domain (LBD) of the receptor to use, the zinc finger array and extent thereof and the stoichiometry and orientation of DNA binding can be empirically determined as exemplified herein for a steroid receptor.

In preferred embodiments the zinc-finger portion of the fusion protein binds to a nucleotide sequence of the formula (GNN)<sub>n</sub>, where G is guanidine, N is any nucleotide and n is an integer from 1 to 6, and typically n is 3 to 6. Preferably, the zinc-finger modular unit is derived from C2H2 zinc-finger peptide. More preferably, the zinc-finger peptide is a C2H2 zinc-finger peptide has at least 90% sequence identity to a human zinc-finger peptide.

### Transcription Regulating Domain (TRD)

The fusion proteins also can include transcription regulating domains. In preferred embodiments, the transcription regulating domain includes a transcription activation domain. Preferably, the transcription regulating domain has at least 90% sequence identity to a mammalian, including human if the fusion protein is intended for human gene therapy, transcription regulating domain to avoid inducing undesirable immunological responses.

The transcription regulating domain can be any such domain known to be a regulator or prepared to regulate eukaryotic transcription. Such TRDs are known, and include, but are not limited to, VP16, VP64, TA2, STAT-6, p65, and derivatives, multimers and combinations thereof that exhibit transcriptional regulation properties. The transcription regulating domain can be derived from an intracellular receptor, such as a nuclear hormone receptor transcription activation (or repression) domain, and is preferably a steroid hormone receptor transcription activation domain or variant thereof that exhibits transcriptional regulation properties. Transcription domains include, but are not limited to, TAF-1, TAF-2, TAU-1, TAU-2, and variants thereof.

The transcription regulating domain may be a viral transcription activation domain or variant thereof. Preferably, the viral transcription regulating domain comprises a VP16 transcription activation domain or variant thereof.

The transcription regulating domain can include a transcription repression domain. Such domains are known, and include, but are not limited to, transcription repression domains selected from among ERD, KRAB, SID, Deacetylase, and derivatives, multimers and combinations thereof, such as KRAB-ERD, SID-ERD, (KRAB)<sub>2</sub>, (KRAB)<sub>3</sub>, KRAB-A, (KRAB-A)<sub>2</sub>, (SID)<sub>2</sub> (KRAB-A)-SID and SID-(KRAB-A).

### Nucleic acid constructs

Also provided are nucleic acid molecules that encode the resulting fusion proteins. The nucleic acids can be included in vectors, suitable for expression of the proteins and/or vectors suitable for gene therapy. Cell containing the vectors are also provided. Typically the cell is a eukaryotic cell. In other embodiments, the cell is a prokaryotic cell.

Also provided are expression cassettes that contain a gene of interest, particularly a gene encoding a therapeutic product, such as an angiogenesis inhibitor, operatively linked to a transcriptional regulatory region or response element, including sequences of nucleic acids to which a fusion proteins provided herein binds and controls transcription, particularly upon binding of a ligand to the LBD of the fusion polypeptide. Such expression cassettes can be included in a vector for gene therapy, and are intended for administration with, before or after, administration of the fusion protein or nucleic acid encoding the fusion protein. Genes of interest for exogenous delivery typically encode therapeutic proteins, such as growth factors, growth factor inhibitors or antagonists, tumor necrosis factor (TNF) inhibitors, anti-tumor agents, angiogenesis agents, anti-angiogenesis agents, clotting factors, apoptotic and other suicide genes.

### Compositions, combinations and kits

Also provided are compositions that contain the fusion proteins or the vectors that encoded the fusion proteins. Combinations of the fusion proteins or nucleic acids encoding the proteins and nucleic acid encoding a targeted gene with regulatory regions selected for activation by the fusion protein are also provided.

Compositions, particularly pharmaceutical compositions containing the fusion polypeptides in a pharmaceutically acceptable carrier are also provided.

Combinations of the expression cassette and fusion polypeptide or nucleic acid molecules, particularly expression vectors that encode the fusion polypeptide are provided. The combinations may include separate

compositions or a single composition containing both elements. Kits containing the combinations and optionally instructions for administration thereof and other reagents used in preparing and administering the combinations are also provided.

5 Hence compositions suitable for gene therapy that contain nucleic acid encoding the fusion protein, typically in a vector suitable for gene therapy are provided. Preferred vectors include viral vectors, preferably adenoviral vectors, and lentiviral vectors. In other embodiments, non-viral delivery systems, including DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA,  $\text{CaPO}_4$  precipitation, gene gun techniques, electroporation, liposomes and lipofection are provided.

10 The compositions suitable for regulating gene expression contain an effective amount of the fusion protein or a polynucleotide encoding the ligand activated transcriptional regulatory fusion protein and a pharmaceutically acceptable excipient. Such compositions can further include a regulatable expression cassette encoding a gene and at least one response element for the gene recognized by the nucleotide binding domain of the fusion polypeptide.

15 The regulatable expression cassette is designed to include a sequence of nucleic acids with which the nucleic acid binding domain of the ligand activated transcriptional regulatory fusion protein interacts. It also preferably includes operatively linked transcriptional regulatory sequences that are regulatable by the TRD of the fusion protein. Typically, the regulatable expression cassette includes 3 to 6 response elements.

### 20 **Methods**

25 Methods for regulating expression of endogenous and exogenous genes are provided. The methods are practiced by administering to a cell a composition that contains an effective amount or concentration of the fusion protein or of nucleic acid molecule, such as a vector that encodes the fusion protein. The nucleic acid binding domain (DBD) of the fusion

protein is selected to bind to a targeted nucleic acid sequence in the genome of the cell or in an exogenously administered nucleic acid molecule, and the transcription regulating domain (TRD) is selected to regulate transcription from a selected promoter, which typically is operatively linked the targeted nucleic acid binding domain. The exogenously administered nucleic acid molecule comprises an expression cassette encoding a gene of interest and operatively linked to a regulatory region that contains elements, such as a promoter and response elements.

As noted the targeted regulatory region and gene of interest may be endogenously present in the cell or separately administered as part of an expression cassette encoding the gene of interest. If separately administered, it is administered as part of a regulatable expression cassette that includes a gene and at least one response element for the gene recognized by the nucleotide binding domain of the fusion protein.

At the same time or at a later time, a composition containing comprising a ligand that binds to the ligand binding domain of the fusion protein is also administered. The ligand can be administered in the same composition as the fusion protein (or encoding nucleic acid molecule) or in a separate composition. The ligand and fusion protein may be administered sequentially, simultaneously or intermittently.

Hence gene therapy is effected by administering a ligand that binds to the LBD of the fusion protein. Preferably the ligand is a non-natural ligand and the LBD has been modified from the native form present in native intracellular receptors to preferentially and selectively interact with the non-natural ligand. Upon administration, the ligand binds to the ligand binding domain of the fusion protein, whereby the DBD of the fusion protein, either as a monomer or dimer, interacts with a targeted gene and transcription of the targeted gene is repressed or activated. As noted, the targeted gene may be an endogenous gene or an exogenously administered gene.

In other embodiments, the methods for regulating gene expression in a cell are effected by administering to the cell a composition containing an effective amount of the nucleic acid molecule that encodes the ligand activated transcriptional regulatory fusion protein, a regulatable expression cassette containing a gene operatively linked to at least one response element for the gene recognized by the nucleotide binding domain of the polypeptide encoded by the polynucleotide, and a pharmaceutically acceptable excipient; and administering to the cell a ligand that binds to the ligand binding domain of the encoded polypeptide, where the nucleotide binding domain of the encoded polypeptide binds to the response element and activates or represses transcription of the gene.

Methods for treating a cellular proliferative disorder by the *ex vivo* introduction of a recombinant expression vector encoding the fusion protein are provided. Cellular proliferative disorder include disorders associated with transcription of a gene at reduced or increased levels.

Administration can of the composition(s) can be effected *in vitro*, *in vivo* or *ex vivo*. One such method includes the removal of a tissue sample from a subject with a disorder, such as a cell proliferative disorder, isolating hematopoietic or other cells from the tissue sample, and contacting isolated cells with the fusion protein or a nucleic acid molecule encoding the fusion protein, and, optionally, a target specific gene. Optionally, the cells can be treated with a growth factor, such as interleukin-2 for example, to stimulate cell growth, before reintroducing the cells into the subject. When reintroduced, the cells specifically target the cell population from which they were originally isolated. In this way, the trans-repressing activity of the zinc finger-nucleotide binding polypeptide may be used to inhibit or suppress undesirable cell proliferation in a subject. Preferably, the subject is a human.

Results exemplified herein demonstrate ligand activated transcription of a targeted gene and demonstrate the utility of the fusion protein

containing a zinc finger DNA binding domain, such as a mammalian C2H2 DNA binding domain, a ligand binding domain from an intracellular receptor, such as an estrogen receptor, and, optionally, a heterologous transcription regulating domain for the purpose of obtaining ligand-dependent control of expression of a transgene introduced into mammalian cells. Hence it is shown herein that heterologous zinc finger domains can be combined with an intracellular receptor to achieve ligand-dependent gene expression of a targeted gene.

#### DESCRIPTION OF THE DRAWINGS

In the drawings, which form a portion of the specification:

**FIGURE 1** is a schematic for the selection strategy for the *in vitro* evolution of the 3 finger protein Zif268, recognizing its natural 9 bp target site (top), into a 6 finger protein, recognizing a desired 18 bp target sequence (bottom).

**FIGURE 2** is a schematic depiction of the functional domains (A-F) of the human estrogen receptor.

**FIGURE 3** is a schematic depiction of the cloning strategy for the construction of the recombinant molecular constructs.

**FIGURE 4** is a schematic map of the expression vector for C7LBDAS based on the plasmid pCDNA3.1.

**FIGURE 5** is a schematic map of the expression vector for C7LBDDBS based on the plasmid pCDNA3.1.

**FIGURE 6** is a schematic map of the expression vector for C7LBDSCS based on the plasmid pCDNA3.1.

**FIGURE 7** is a schematic map of the expression vector for C7LBDAL based on the plasmid pCDNA3.1.

**FIGURE 8** is a schematic map of the expression vector for C7LBDDBL based on the plasmid pCDNA3.1.

**FIGURE 9** is a schematic map of the expression vector for C7LBDCL based on the plasmid pCDNA3.1.

**FIGURE 10** is a schematic summary of the structure of several embodiments of the recombinant molecular construct and the nucleotide sequences of the DNA binding regions of zinc finger domains C7, E2C and 2C7.

**FIGURE 11** is a schematic map of the expression vector for E2CLBDAS based on the plasmid pCDNA3.1.

**FIGURE 12** is a schematic map of the expression vector for E2CLBDBS based on the plasmid pCDNA3.1.

**FIGURE 13** is a schematic diagram of the constructs C7LBDA2A2, C7LBDB2A2, C7LBDBS-STAT6, C7LBDBSVP16 (SEQ ID NO: 16), AND C7LBDBSNLSVP16.

**FIGURE 14** is a schematic restriction map of constructs comprising RXR and ecdysone (EcR) ligand binding domains used in heterodimers.

**FIGURE 15** is a schematic depiction of the cloning strategy for the construction of the 2C7LBD recombinant molecular constructs.

**FIGURE 16** is a schematic map of the expression vector for 2C7LBDAS based on the plasmid pCDNA3.1.

**FIGURE 17** is a schematic map of the expression vector for 2C7LBDBS based on the plasmid pCDNA3.1.

**FIGURE 18** is a schematic map of the expression vector for 2C7LBDCS based on the plasmid pCDNA3.1.

**FIGURE 19** is a schematic map of the expression vector for LBDASNLSVP16 (SEQ ID NO: 13), based on the plasmid pCDNA3.1.

**FIGURE 20** is a schematic map of the expression vector for C7LBDBSVP16 based on the plasmid pCDNA3.1.

**FIGURE 21** is a schematic map of the expression vector for C7LBDBSG521R (SEQ ID NO: 15), based on the plasmid pCDNA3.1.

**FIGURE 22** is a schematic map of the expression vector for C7LBDBSG400V (SEQ ID NO: 14), based on the plasmid pCDNA3.1.

**FIGURE 23** shows A: an inducible promoter based on binding sites for the 3 Finger protein N1. The promoter contains 5 direct repeats of N1 sites

spaced by 3 bp; the spacing between the 5 repeats is 6 bp. Bottom: Luciferase assay. HeLa cells were cotransfected with plasmids encoding the indicated fusion proteins and the N1 reporter construct. Twenty four hours later, the cells were treated with 10 nM RU486 (B) or 100nM Tamoxifen, C respectively. Forty-eight hours post transfection, cell extracts were assayed for luciferase activity.

**FIGURE 24** shows an inducible promoter based on binding sites for the 3 Finger protein B3. A: The promoter contains 5 direct repeats of B3 sites spaced by 3 bp; the spacing between the 5 repeats is 6 bp. Bottom: Luciferase assay. HeLa cells were cotransfected with plasmids encoding the indicated fusion proteins and the B3 reporter construct. At 24 h later, the cells were treated with 10 nM RU486 (B), or 100 nM Tamoxifen (C), respectively. At 48 h post transfection, cell extracts were assayed for luciferase activity.

**FIGURE 25** is a graphical depiction of the results of luciferase assay showing the RU486-induced formation of functional VP64-C7-PR/VP64-CF2-PR heterodimers. HeLa cells were cotransfected with the corresponding effector plasmids and TATA reporter plasmids (C7/CF2-dr0, C7 site 5' to a CF2 site, direct "repeat", no spacing; C7/C7-dr0, 2 C7 sites, direct repeat, no spacing). At 24 h later, the cells were treated with 10 nM RU486. At 48 h post transfection, cell extracts were assayed for luciferase activity.

**FIGURE 26** shows a restriction map for the plasmid designated pAvCVI $\alpha$ .

**FIGURE 27** shows a restriction map for the plasmid designated pSQ3.

## DETAILED DESCRIPTION

### I. DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as is commonly understood by one of skill in the art to which this invention belongs. All patents, applications, published applications and other publications and sequences from

GenBank and other data bases referred to anywhere in the disclosure herein are incorporated by reference in their entirety.

As used herein, the ligand binding domain (LBD) of the fusion proteins provided herein refers to the portion of the fusion protein responsible for binding to a selected ligand. The LBD optionally and preferably includes dimerization and inactivation functions. The LBDs in the proteins herein are derived from the 300 amino acid carboxyl-terminal half of intracellular receptors, particularly those that are members of the steroid hormone nuclear receptor superfamily. It is the portion of the receptor protein with which a ligand interacts thereby inducing a cascade of events leading to the specific association of an activated receptor with regulatory elements of target genes. In these receptors the LBD includes the hormone binding function, the inactivation function, such as through interactions with heat shock proteins (hsp), and dimerization function. The LBDs used herein include such LBDs and modified derivatives thereof, particularly forms with altered ligand specificity.

As used herein, the transcription regulating domain (TRD) refers to the portion of the fusion polypeptide provided herein that functions to regulate gene transcription. Exemplary and preferred transcription repressor domains are ERD, KRAB, SID, Deacetylase, and derivatives, multimers and combinations thereof such as KRAB-ERD, SID-ERD, (KRAB)<sub>2</sub>, (KRAB)<sub>3</sub>, KRAB-A, (KRAB-A)<sub>2</sub>, (SID)<sub>2</sub> (KRAB-A)-SID and SID-(KRAB-A).

As used herein, the DNA binding domain (DBD), or alternatively the nucleic acid (or nucleotide) binding domain, refers to the portion of the fusion polypeptide provided herein that provides specific nucleic acid binding capability. The use of the abbreviation DBD is not meant to limit it to DNA binding domains, but is also intended to include polypeptides that bind to RNA. The nucleic acid binding domain functions to target the protein to specific genes by virtue of the specificity of the interaction of the TRD region for nucleotide sequences operatively linked to the

transcriptional apparatus of a gene. The DBD targets the fusion protein to the selected targeted gene or genes, which gene(s) may be endogenous or exogenously added.

As used herein, operatively linked means that elements of the fusion polypeptide, for example, are linked such that each perform or function as intended. For example, the repressor is attached to the binding domain in such a manner that, when bound to a target nucleotide via that binding domain, the repressor acts to inhibit or prevent transcription. Linkage between and among elements may be direct or indirect, such as via a linker. The elements are not necessarily adjacent. Hence a repressor domain of a TRD can be linked to a DNA binding domain using any linking procedure well known in the art. It may be necessary to include a linker moiety between the two domains. Such a linker moiety is typically a short sequence of amino acid residues that provides spacing between the domains. So long as the linker does not interfere with any of the functions of the binding or repressor domains, any sequence can be used.

As used herein, a fusion protein is a protein that contains portions or fragments of two or more naturally-occurring proteins operatively joined or linked to form the fusion protein in which each fragment retains a function or a modified function exhibited by the naturally occurring proteins. The fragments from the naturally occurring protein may be modified to alter the original properties.

As used herein, modified, modification, mutant or other such terms refers to an alteration of the domain in question from its naturally occurring wild-type form, and includes primary sequence changes.

As used herein, "modulating" envisions the inhibition or suppression of expression from a promoter containing a zinc finger-nucleotide binding motif when it is over-activated, or augmentation or enhancement of expression from such a promoter when it is under-activated.

As used herein, steroid hormone receptor superfamily refers to the superfamily of intracellular receptors that are steroid receptors. Representative examples of such receptors include, but are not limited to, the estrogen, progesterone, glucocorticoid- $\alpha$ , glucocorticoid- $\beta$ , mineralocorticoid, androgen, thyroid hormone, retinoic acid, retinoid X, Vitamin D, COUP-TF, ecdysone, Nurr-1 and orphan receptors.

As used herein, the amino acids, which occur in the various amino acid sequences appearing herein, are identified according to their well-known, three-letter or one-letter abbreviations. The nucleotides, which occur in the various DNA fragments, are designated with the standard single-letter designations used routinely in the art.

In a peptide or protein, suitable conservative substitutions of amino acids are known to those of skill in this art and may be made generally without altering the biological activity of the resulting molecule. Those of skill in this art recognize that, in general, single amino acid substitutions in non-essential regions of a polypeptide do not substantially alter biological activity (see, e.g., Watson et al. *Molecular Biology of the Gene*, 4th Edition, 1987, The Bejacmin/Cummings Pub. co., p.224).

As used herein, a delivery plasmid is a plasmid vector that carries or delivers nucleotide acids encoding a therapeutic gene or gene that encodes a therapeutic product or a precursor thereof or a regulatory gene or other factor that results in a therapeutic effect when delivered *in vivo* in or into a cell line, such as, but not limited to a packaging cell line, to propagate therapeutic viral vectors.

As used herein, "recombinant expression vector" or "expression vector" refers to a plasmid, virus or other vehicle known in the art that has been manipulated by insertion or incorporation of heterologous DNA, such as nucleic acid encoding the fusion proteins herein or expression cassettes provided herein. Such expression vectors contain a promotor sequence for efficient transcription of the inserted nucleic acid in a cell. The expression vector typically contains an origin of replication, a

promoter, as well as specific genes that permit phenotypic selection of transformed cells.

As used herein, a DNA or nucleic acid homolog refers to a nucleic acid that includes a preselected conserved nucleotide sequence, such as a sequence encoding a therapeutic polypeptide. By the term "substantially homologous" is meant having at least 80%, preferably at least 90%, most preferably at least 95% homology therewith or a less percentage of homology or identity and conserved biological activity or function.

As used herein, "host cells" are cells in which a vector can be propagated and its DNA expressed. The term also includes any progeny of the subject host cell. It is understood that all progeny may not be identical to the parental cell since there may be mutations that occur during replication. Such progeny are included when the term "host cell" is used. Methods of stable transfer where the foreign DNA is continuously maintained in the host are known in the art.

The terms "homology" and "identity" are often used interchangeably. In this regard, percent homology or identity may be determined, for example, by comparing sequence information using a GAP computer program. The GAP program uses the alignment method of Needleman and Wunsch ((1970) *J. Mol. Biol.* 48:443), as revised by Smith and Waterman ((1981) *Adv. Appl. Math.* 2:482). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program may include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) and the weighted comparison matrix of Gribskov *et al.* (1986) *Nucl. Acids Res.* 14:6745, as described by Schwartz and Dayhoff, eds., *ATLAS OF PROTEIN SEQUENCE AND STRUCTURE*, National Biomedical Research Foundation, pp. 353-358 (1979); (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Whether any two nucleic acid molecules have nucleotide sequences that are at least 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% "identical" can be determined using known computer algorithms such as the "FAST A" program, using for example, the default parameters as in Pearson *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:2444. Alternatively the BLAST function of the National Center for Biotechnology Information database may be used to determine identity

In general, sequences are aligned so that the highest order match is obtained. "Identity" *per se* has an art-recognized meaning and can be calculated using published techniques. (See, *e.g.*: *Computational Molecular Biology*, Lesk, A.M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D.W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A.M., and Griffin, H.G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991). While there exist a number of methods to measure identity between two polynucleotide or polypeptide sequences, the term "identity" is well known to skilled artisans (Carillo *et al.* (1988) *SIAM J Applied Math* 48:1073). Methods commonly employed to determine identity or similarity between two sequences include, but are not limited to, those disclosed in Guide to Huge Computers, Martin J. Bishop, ed., Academic Press, San Diego, 1994, and Carillo *et al.* (1988) *SIAM J Applied Math* 48:1073. Methods to determine identity and similarity are codified in computer programs. Preferred computer program methods to determine identity and similarity between two sequences include, but are not limited to, GCG program package (Devereux, J., *et al.*, *Nucleic Acids Research* 12(II):387 (1984)), BLASTP, BLASTN, FASTA (Atschul, S.F., *et al.*, *J Molec Biol* 215:403 (1990)).

Therefore, as used herein, the term "identity" represents a comparison between a test and a reference polypeptide or polynucleotide. For example, a test polypeptide may be defined as any polypeptide that is 90% or more identical to a reference polypeptide. As used herein, the term at least "90% identical to" refers to percent identities from 90 to 99.99 relative to the reference polypeptides. Identity at a level of 90% or more is indicative of the fact that, assuming for exemplification purposes a test and reference polynucleotide length of 100 amino acids are compared. No more than 10% (i.e., 10 out of 100) amino acids in the test polypeptide differs from that of the reference polypeptides. Similar comparisons may be made between a test and reference polynucleotides. Such differences may be represented as point mutations randomly distributed over the entire length of an amino acid sequence or they may be clustered in one or more locations of varying length up to the maximum allowable, e.g. 10/100 amino acid difference (approximately 90% identity). Differences are defined as nucleic acid or amino acid substitutions, or deletions.

As used herein, primer refers to an oligonucleotide containing two or more deoxyribonucleotides or ribonucleotides, preferably more than three, from which synthesis of a primer extension product can be initiated. For purposes herein, a primer of interest is one that is substantially complementary to a zinc finger-nucleotide binding protein strand, but also can introduce mutations into the amplification products at selected residue sites. Experimental conditions conducive to synthesis include the presence of nucleoside triphosphates and an agent for polymerization and extension, such as DNA polymerase, and a suitable buffer, temperature and pH.

As used herein, genetic therapy involves the transfer of heterologous DNA to the certain cells, target cells, of a mammal, particularly a human, with a disorder or conditions for which such therapy is sought. The DNA is introduced into the selected target cells in a

manner such that the heterologous DNA is expressed and a therapeutic product encoded thereby is produced. Alternatively, the heterologous DNA may in some manner mediate expression of DNA that encodes the therapeutic product, or it may encode a product, such as a peptide or RNA that in some manner mediates, directly or indirectly, expression of a therapeutic product. Genetic therapy may also be used to deliver nucleic acid encoding a gene product that replaces a defective gene or supplements a gene product produced by the mammal or the cell in which it is introduced. The introduced nucleic acid may encode a therapeutic compound, such as a growth factor inhibitor thereof, or a tumor necrosis factor or inhibitor thereof, such as a receptor therefor, that is not normally produced in the mammalian host or that is not produced in therapeutically effective amounts or at a therapeutically useful time. The heterologous DNA encoding the therapeutic product may be modified prior to introduction into the cells of the afflicted host in order to enhance or otherwise alter the product or expression thereof. Genetic therapy may also involve delivery of an inhibitor or repressor or other modulator of gene expression.

As used herein, heterologous DNA is DNA that encodes RNA and proteins that are not normally produced *in vivo* by the cell in which it is expressed or that mediates or encodes mediators that alter expression of endogenous DNA by affecting transcription, translation, or other regulatable biochemical processes. Heterologous DNA may also be referred to as foreign DNA. Any DNA that one of skill in the art would recognize or consider as heterologous or foreign to the cell in which is expressed is herein encompassed by heterologous DNA. Examples of heterologous DNA include, but are not limited to, DNA that encodes traceable marker proteins, such as a protein that confers drug resistance, DNA that encodes therapeutically effective substances, such as anti-cancer agents, enzymes and hormones, and DNA that encodes other types of proteins, such as antibodies. Antibodies that are encoded by

heterologous DNA may be secreted or expressed on the surface of the cell in which the heterologous DNA has been introduced.

Hence, herein heterologous DNA or foreign DNA, includes a DNA molecule not present in the exact orientation and position as the counterpart DNA molecule found in the genome. It may also refer to a DNA molecule from another organism or species (*i.e.*, exogenous).

As used herein, a therapeutically effective product is a product that is encoded by heterologous nucleic acid, typically DNA, that, upon introduction of the nucleic acid into a host, a product is expressed that ameliorates or eliminates the symptoms, manifestations of an inherited or acquired disease or that cures the disease.

Typically, DNA encoding a desired gene product is cloned into a plasmid vector and introduced by routine methods, such as calcium-phosphate mediated DNA uptake (see, (1981) *Somat. Cell. Mol. Genet.* 7:603-616) or microinjection, into producer cells, such as packaging cells.

After amplification in producer cells, the vectors that contain the heterologous DNA are introduced into selected target cells.

As used herein, an expression or delivery vector refers to any plasmid or virus into which a foreign or heterologous DNA may be inserted for expression in a suitable host cell — *i.e.*, the protein or polypeptide encoded by the DNA is synthesized in the host cell's system. Vectors capable of directing the expression of DNA segments (genes) encoding one or more proteins are referred to herein as "expression vectors." Also included are vectors that allow cloning of cDNA (complementary DNA) from mRNAs produced using reverse transcriptase.

As used herein, a gene refers to a nucleic acid molecule whose nucleotide sequence encodes an RNA or polypeptide. A gene can be either RNA or DNA. Genes may include regions preceding and following the coding region (leader and trailer) as well as intervening sequences (introns) between individual coding segments (exons).

As used herein, isolated with reference to a nucleic acid molecule or polypeptide or other biomolecule means that the nucleic acid or polypeptide has separated from the genetic environment from which the polypeptide or nucleic acid were obtained. It may also mean altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated," but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated. Also intended as an "isolated polypeptide" or an "isolated polynucleotide" are polypeptides or polynucleotides that have been purified, partially or substantially, from a recombinant host cell or from a native source. For example, a recombinantly produced version of a compound can be substantially purified by the one-step method described in Smith *et al.* (1988) *Gene* 67:31-40. The terms isolated and purified are sometimes used interchangeably.

Thus, by "isolated" the nucleic acid is free of the coding sequences of those genes that, in a naturally-occurring genome immediately flank the gene encoding the nucleic acid of interest. Isolated DNA may be single-stranded or double-stranded, and may be genomic DNA, cDNA, recombinant hybrid DNA, or synthetic DNA. It may be identical to a native DNA sequence, or may differ from such sequence by the deletion, addition, or substitution of one or more nucleotides.

Isolated or purified as it refers to preparations made from biological cells or hosts means any cell extract containing the indicated DNA or protein including a crude extract of the DNA or protein of interest. For example, in the case of a protein, a purified preparation can be obtained following an individual technique or a series of preparative or biochemical techniques and the DNA or protein of interest can be present at various degrees of purity in these preparations. The procedures may include for example, but are not limited to, ammonium sulfate fractionation, gel

filtration, ion exchange change chromatography, affinity chromatography, density gradient centrifugation and electrophoresis.

A preparation of DNA or protein that is "substantially pure" or "isolated" should be understood to mean a preparation free from naturally occurring materials with which such DNA or protein is normally associated in nature. "Essentially pure" should be understood to mean a "highly" purified preparation that contains at least 95% of the DNA or protein of interest.

A cell extract that contains the DNA or protein of interest should be understood to mean a homogenate preparation or cell-free preparation obtained from cells that express the protein or contain the DNA of interest. The term "cell extract" is intended to include culture media, especially spent culture media from which the cells have been removed.

As used herein, "modulate" refers to the suppression, enhancement or induction of a function. For example, zinc finger-nucleic acid binding domains and variants thereof may modulate a promoter sequence by binding to a motif within the promoter, thereby enhancing or suppressing transcription of a gene operatively linked to the promoter cellular nucleotide sequence. Alternatively, modulation may include inhibition of transcription of a gene where the zinc finger-nucleotide binding polypeptide variant binds to the structural gene and blocks DNA dependent RNA polymerase from reading through the gene, thus inhibiting transcription of the gene. The structural gene may be a normal cellular gene or an oncogene, for example. Alternatively, modulation may include inhibition of translation of a transcript.

As used herein, "inhibit" refers to the suppression of the level of activation of transcription of a structural gene operably linked to a promoter. For example, for the methods herein the gene includes a zinc finger-nucleotide binding motif.

As used herein, a transcriptional regulatory region refers to a region that drives gene expression in the target cell. Transcriptional regulatory

regions suitable for use herein include but are not limited to the human cytomegalovirus (CMV) immediate-early enhancer/promoter, the SV40 early enhancer/promoter, the JC polyomavirus promoter, the albumin promoter, PGK and the  $\alpha$ -actin promoter coupled to the CMV enhancer.

5 As used herein, a promoter region of a gene includes the regulatory elements that typically lie 5' to a structural gene. If a gene is to be activated, proteins known as transcription factors attach to the promoter region of the gene. This assembly resembles an "on switch" by enabling an enzyme to transcribe a second genetic segment from DNA into RNA. In most cases the resulting RNA molecule serves as a template for synthesis of a specific protein; sometimes RNA itself is the final product. The promoter region may be a normal cellular promoter or, for example, an onco-promoter. An onco-promoter is generally a virus-derived promoter. Viral promoters to which zinc finger binding polypeptides may be targeted include, but are not limited to, retroviral long terminal repeats (LTRs), and *Lentivirus* promoters, such as promoters from human T-cell lymphotropic virus (HTLV) 1 and 2 and human immunodeficiency virus (HIV) 1 or 2.

10 As used herein, "effective amount" includes that amount that results in the deactivation of a previously activated promoter or that amount that results in the inactivation of a promoter containing a zinc finger-nucleotide binding motif, or that amount that blocks transcription of a structural gene or translation of RNA. The amount of zinc finger derived-nucleotide binding polypeptide required is that amount necessary to either displace a native zinc finger-nucleotide binding protein in an existing protein/promoter complex, or that amount necessary to compete with the native zinc finger-nucleotide binding protein to form a complex with the promoter itself. Similarly, the amount required to block a structural gene or RNA is that amount which binds to and blocks RNA polymerase from reading through on the gene or that amount which inhibits translation, respectively. Preferably, the method is performed

intracellularly. By functionally inactivating a promoter or structural gene, transcription or translation is suppressed. Delivery of an effective amount of the inhibitory protein for binding to or "contacting" the cellular nucleotide sequence containing the zinc finger-nucleotide binding protein motif, can be accomplished by one of the mechanisms described herein, such as by retroviral vectors or liposomes, or other methods well known in the art.

As used herein, "truncated" refers to a zinc finger-nucleotide binding polypeptide derivative that contains less than the full number of zinc fingers found in the native zinc finger binding protein or that has been deleted of non-desired sequences. For example, truncation of the zinc finger-nucleotide binding protein TFIIIA, which naturally contains nine zinc fingers, might be a polypeptide with only zinc fingers one through three. Expansion refers to a zinc finger polypeptide to which additional zinc finger modules have been added. For example, TFIIIA may be extended to 12 fingers by adding 3 zinc finger domains. In addition, a truncated zinc finger-nucleotide binding polypeptide may include zinc finger modules from more than one wild type polypeptide, thus resulting in a "hybrid" zinc finger-nucleotide binding polypeptide.

As used herein, "mutagenized" refers to a zinc finger derived-nucleotide binding polypeptide that has been obtained by performing any of the known methods for accomplishing random or site-directed mutagenesis of the DNA encoding the protein. For instance, in TFIIIA, mutagenesis can be performed to replace nonconserved residues in one or more of the repeats of the consensus sequence. Truncated zinc finger-nucleotide binding proteins can also be mutagenized.

As used herein, a polypeptide "variant" or "derivative" refers to a polypeptide that is a mutagenized form of a polypeptide or one produced through recombination but that still retains a desired activity, such as the ability to bind to a ligand or a nucleic acid molecule or to modulate transcription.

As used herein, a zinc finger-nucleotide binding polypeptide "variant" or "derivative " refers to a polypeptide that is a mutagenized form of a zinc finger protein or one produced through recombination. A variant may be a hybrid that contains zinc finger domain(s) from one protein linked to zinc finger domain(s) of a second protein, for example. The domains may be wild type or mutagenized. A "variant " or "derivative" includes a truncated form of a wild type zinc finger protein, which contains less than the original number of fingers in the wild type protein. Examples of zinc finger-nucleotide binding polypeptides from which a derivative or variant may be produced include TFIIIA and zif268. Similar terms are used to refer to "variant" or "derivative " nuclear hormone receptors and "variant" or "derivative " transcription effector domains.

As used herein a "zinc finger-nucleotide binding motif" refers to any two or three-dimensional feature of a nucleotide segment to which a zinc finger-nucleotide binding derivative polypeptide binds with specificity. Included within this definition are nucleotide sequences, generally of five nucleotides or less, as well as the three dimensional aspects of the DNA double helix, such as, but are not limited to, the major and minor grooves and the face of the helix. The motif is typically any sequence of suitable length to which the zinc finger polypeptide can bind. For example, a three finger polypeptide binds to a motif typically having about 9 to about 14 base pairs. Preferably, the recognition sequence is at least about 16 base pairs to ensure specificity within the genome. Therefore, zinc finger-nucleotide binding polypeptides of any specificity are provided. The zinc finger binding motif can be any sequence designed empirically or to which the zinc finger protein binds. The motif may be found in any DNA or RNA sequence, including regulatory sequences, exons, introns, or any non-coding sequence.

As used herein, the terms "pharmaceutically acceptable", "physiologically tolerable" and grammatical variations thereof, as they

refer to compositions, carriers, diluents and reagents, are used interchangeably and represent that the materials are capable of administration to or upon a human without the production of undesirable physiological effects such as nausea, dizziness, gastric upset and the like which would be to a degree that would prohibit administration of the composition.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting between different genetic environments another nucleic acid to which it has been operatively linked. Preferred vectors are those capable of autonomous replication and expression of structural gene products present in the DNA segments to which they are operatively linked. Vectors, therefore, preferably contain the replicons and selectable markers described earlier.

As used herein with regard to nucleic acid molecules, including DNA fragments, the phrase "operatively linked" means the sequences or segments have been covalently joined, preferably by conventional phosphodiester bonds, into one strand of DNA, whether in single or double stranded form such that operatively linked portions functions as intended. The choice of vector to which transcription unit or a cassette provided herein is operatively linked depends directly, as is well known in the art, on the functional properties desired, *e.g.*, vector replication and protein expression, and the host cell to be transformed, these being limitations inherent in the art of constructing recombinant DNA molecules.

As used herein, a sequence of nucleotides adapted for directional ligation, *i.e.*, a polylinker, is a region of the DNA expression vector that (1) operatively links for replication and transport the upstream and downstream translatable DNA sequences and (2) provides a site or means for directional ligation of a DNA sequence into the vector. Typically, a directional polylinker is a sequence of nucleotides that defines two or more restriction endonuclease recognition sequences, or restriction sites. Upon restriction cleavage, the two sites yield cohesive termini to which a

translatable DNA sequence can be ligated to the DNA expression vector. Preferably, the two restriction sites provide, upon restriction cleavage, cohesive termini that are non-complementary and thereby permit directional insertion of a translatable DNA sequence into the cassette. In one embodiment, the directional ligation means is provided by nucleotides present in the upstream translatable DNA sequence, downstream translatable DNA sequence, or both. In another embodiment, the sequence of nucleotides adapted for directional ligation comprises a sequence of nucleotides that defines multiple directional cloning means. Where the sequence of nucleotides adapted for directional ligation defines numerous restriction sites, it is referred to as a multiple cloning site.

As used herein, a secretion signal is a leader peptide domain of a protein that targets the protein to the periplasmic membrane of gram negative bacteria. A preferred secretion signal is a pelB secretion signal. The predicted amino acid residue sequences of the secretion signal domain from two pelB gene product variants from *Erwinia carotova* are described in Lei, *et al.* (*Nature*, 331:543-546, 1988). The leader sequence of the pelB protein has previously been used as a secretion signal for fusion proteins (Better *et al.* (1988) *Science* 240:1041-1043; Sastry *et al.* (1989) *Proc. Natl. Acad. Sci. USA* 86:5728-5732; and Mullinax *et al.* (1990) *Proc. Natl. Acad. Sci. USA*, 87:8095-8099). Amino acid residue sequences for other secretion signal polypeptide domains from *E. coli* are known (see, *e.g.*, Oliver, In Neidhard, F.C. (ed.), *Escherichia coli* and *Salmonella Typhimurium*, American Society for Microbiology, Washington, D.C., 1:56-69 (1987)).

As used herein, ligand refers to any compound interacts with the ligand binding domain of a receptor and modulate its activity; ligands typically activate receptors. Ligand can also include compounds that activate the receptor without binding. A natural ligand is a compound that normally interacts with the receptor.

As used herein, anti-hormones are compounds that are antagonists of the naturally-occurring receptor. The anti-hormone is opposite in activity to a hormone.

As used herein, non-natural ligands or non-native ligands refer to compounds that are normally are not found in mammals, such as humans, that bind to or interact with the ligand binding domain of a receptor. Hence, the term "non-native ligands" refers to those ligands that are not naturally found in the specific organism (man or animal) in which gene therapy is contemplated. For example, certain insect hormones such as ecdysone are not found in humans. As such ecdysone is non-native hormone to an animal, such as a human.

As used herein, "cell-proliferative disorder" denotes malignant as well as non-malignant disorders in which cell populations morphologically appear to differ from the surrounding tissue. The cell-proliferative disorder may be a transcriptional disorder that results in an increase or a decrease in gene expression level. The cause of the disorder may be of cellular origin or viral origin. Gene therapy using a zinc finger-nucleotide binding polypeptide can be used to treat a virus-induced cell proliferative disorder in a human, for example, as well as in a plant. Treatment can be prophylactic in order to make a plant cell, for example, resistant to a virus, or therapeutic, in order to ameliorate an established infection in a cell, by preventing production of viral products.

As used herein, "cellular nucleotide sequence" refers to a nucleotide sequence that is present within a cell. It is not necessary that the sequence be a naturally occurring sequence of the cell. For example, a retroviral genome that is integrated within a host's cellular DNA, would be considered a "cellular nucleotide sequence". The cellular nucleotide sequence can be DNA or RNA and includes introns and exons, DNA and RNA. The cell and/or cellular nucleotide sequence can be prokaryotic or eukaryotic, including a yeast, virus, or plant nucleotide sequence.

As used herein, administration of a therapeutic composition can be effected by any means, and includes, but is not limited to, subcutaneous, intravenous, intramuscular, intrasternal, infusion techniques, intraperitoneally administration and parenteral administration.

## II. Fusion Protein

### A. General

The fusion protein is constructed to include a ligand binding domain and a nucleic acid binding domain; the nucleic acid binding domain is not derived from the same receptor as the ligand binding domain. Inclusion of these two domains permits sequence specific binding to target nucleic acid sequences present in endogenous or exogenous nucleic acid molecules. It also provides ligand-dependent control of such sequence-specific binding. The fusion protein can also include a transcription regulating domain that serves to enhance, suppress or activate expression of an endogenous or exogenous gene. Such transcriptional control is also ligand dependent.

The nucleic acid binding domain (the DBD) includes one or more zinc finger peptide modular units, and typically a plurality of such units joined to provide a peptide designed to bind to the regulatory region in a targeted gene. Zinc fingers provide a means to design DBDs of a desired specificity.

The fusion protein also includes a LBD that derived from an intracellular receptor, preferably a hormone receptor, more preferably a steroid receptor. The LBD can be modified to have altered ligand specificity so that endogenous or natural ligands do not interact with it, but non-natural ligands do. The fusion protein also can include a transcription regulating domain (TRD) that regulates transcription of the targeted gene(s). In some embodiments, the TRD can repress transcription of an endogenous gene; in others it can activate expression of an endogenous or exogenous gene.

Hence the fusion protein is made by operably linking a LBD domain from an intracellular receptor to a one or more zinc finger domains, selected to bind to a targeted gene. A transcription regulating domain can also be operably linked. This is accomplished by any method known to those of skill in the art. Generally the fusion protein is produced by expressing nucleic acid encoding the fusion protein.

#### 1. Ligand Binding Domain (LBD)

The ligand binding domain is derived from an intracellular receptor, and is preferably derived from a nuclear hormone receptor. The LBD of an intracellular receptor includes the approximately 300 amino acids from the carboxy terminal, which can be used with or without modification.

By mutation of a small number of residues ligand specificity can be altered. The ligand binding domain can be modified, such as by truncation or point mutation to alter its ligand specificity permitting gene regulation by non-natural or non-native ligands.

Exemplary hormone receptors are steroid receptors, which are well known in the art. Exemplary and preferred steroid receptors include estrogen and progesterone receptors and variants thereof. Of particular interest are ligand binding domains that exhibit altered ligand specificity so that the LBD does not respond to the natural hormone, but rather to a drug, such as RU486, or other inducer. Means to modify and test the specificity of ligand binding domains and to identify ligands therefor are known (see, U.S. Patent No. 5,874,534; U.S. Patent No. 5,935,934; and International PCT application No. 98/18925, which is based on U.S. provisional application Serial No. 60/029,964; International PCT application No. 96/40911, which is based on U.S. application Serial No. 08/479,913).

The LBD can be modified by deletion of from about 1 up to about 150, typically 120, amino acids on the carboxyl terminal end of the receptor from which the LBD derives. Systematic deletion of amino acids and subsequent testing of the ligand specificity and of the resulting LBD

can be used to empirically identify mutations that lead to modified LBDs that have desired properties, such as preferential interaction with non-natural ligands. Exemplary mutations are described in the Examples herein, and also are known to those of skill in the art (see, *e.g.*, U.S. Patent No. 5,874,534; U.S. Patent No. 5,935,934; U.S. Patent No. 5,364,791; and International PCT application No. 98/18925, which is based on U.S. provisional application Serial No. 60/029,964; International PCT application No. 96/40911, which is based on U.S. application Serial No. 08/479,913) and references cited therein. Hence a LBD or modified form thereof prepared by known methods is obtained and operably linked to a DBD; a TRD is also linked as needed.

## 2. Nucleic Acid Binding Domain (DBD)

Zinc fingers are modular nucleic acid binding peptides. The zinc fingers, or modules thereof, or variant thereof can be used to construct fusion proteins that specifically interact with targeted sequences. Zinc fingers are ubiquitous proteins, and many are well-characterized. For example, methods and rules for preparation and selection of zinc fingers based upon the C2H2 class of zinc fingers with unique specificity are known (see, *e.g.*, International PCT application No. WO 98/54311 and International PCT application No. 95/19431; see, also U.S. Patent No. 5,789,538; Beerli *et al.* (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96:2758-2763; Beerli *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633; see, also U.S. application Serial No. 09/173,941, filed 16 October, 1998, published as International PCT application No. WO 00/23464). Exemplary targeting sequences are provided herein.

Furthermore, other zinc fingers can be similarly identified and the rules known for the C2H2 can be applied to modification of the specificity of such zinc fingers or alternative rules unique to each class can be deduced in a similar manner.

The advantage of using zinc fingers for targeting of the ligand-dependent transcription regulating fusion proteins provided herein is the

ability to construct zinc fingers with unique specificity. This permits targeting and ligand-dependent control of expression of specific endogenous genes and also ligand-dependent control of exogenously administered genes, such as genes that encode therapeutic products.

5 Zinc fingers and modular units thereof can be obtained or prepared by any method known to those of skill in the art. As discussed herein, a plethora of zinc fingers, including synthetic zinc fingers having a variety of sequence specificities are known, as are means for combining the modular domains to produce a resulting peptide that binds to any desired  
10 target sequence of nucleic acids. Rules for creating zinc fingers of desired specificity are known and can be deduced by methods used by those of skill in the art (see, *e.g.*, (see, *e.g.*, International PCT application No. WO 98/54311, which is based on U.S. application Serial No. 08/863,813; International PCT application No. 95/19431, which is based  
15 on U.S. application Serial Nos. 08/183,119 and 08/312,604).

For example, zinc finger variants can be prepared by identifying a zinc finger or modular unit thereof, creating an expression library, such as a phage display library (see, *e.g.*, International PCT application No. WO 98/54311, Barbas *et al.* (1991) *Methods* 2:119; Barbas *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:4457), encoding polypeptide variants of  
20 the zinc finger or modular unit thereof, expressing the library in a host and screening for variant peptides having a desired specificity. Zinc fingers may also be constructed by combining amino acids (or encoding nucleic acids) according to the known rules of binding specificity and, if  
25 necessary, testing or screening the resulting peptides to ensure the peptide has a desired specificity. Because of the modular nature of zinc fingers, where each module can be prepared to bind to three nucleotide sequence, peptides of any specificity can be prepared from the modules. The number of modules used depends upon the specificity of gene  
30 targeting desired. Modular units are combined; spacers (*i.e.* TGEKP,

TGQKP) required to maintain spacing and conformational features of the modular domains are included in the peptide (see, *e.g.*, WO 98/54311).

**a. Zinc fingers as DBDs and zinc finger modular units**

The nucleic acid binding domain in the fusion protein includes zinc finger modular domains and is designed to bind to a target nucleic acid sequence present in an endogenous gene or in an exogenous gene that is administered in combination with the fusion protein or nucleic acid encoding the fusion protein.

Zinc fingers are among the most common and ubiquitous nucleic acid binding proteins. Any zinc finger polypeptide or modular unit thereof is contemplated; preferably the domain is non-immunogenic in the host for which the fusion protein is intended. For human therapy, the zinc finger DBD preferably is selected from human zinc protein modular units or variants thereof.

For purposes herein, the zinc finger used generally is other than the naturally-occurring zinc finger present in the intracellular receptor from which the ligand binding domain is derived. Typically the fusion protein is produced by replacing the native zinc finger present in the receptor with the selected zinc finger designed to interact with a targeted nucleic acid regulatory region. In addition, the zinc fingers can be designed by selection of appropriate modular units to have specificity for a targeted gene, thereby providing a precise means to modulate expression of a targeted gene.

Naturally occurring zinc finger proteins generally contain multiple repeats of the zinc finger motif. This modular nature is unique among the different classes of DNA binding proteins. Wild type zinc finger proteins are made up of from two to as many as 37 modular tandem repeats, with each repeat forming a "finger" holding a zinc atom in tetrahedral coordination by means of a pair of conserved cysteines and a pair of conserved histidines. Generally each finger also contains conserved hydrophobic amino acids that interact to form a hydrophobic core that

helps the module maintain its shape. Polydactyl arrays of as many as 37 zinc finger domains allow this recognition domain to recognize extended asymmetric sequences. Any such zinc finger or combinations of modular units thereof is intended for use herein.

5 A zinc finger-nucleotide binding peptide domain contains a unique heptamer (contiguous sequence of 7 amino acid residues) within the  $\alpha$ -helical domain of the polypeptide, which heptameric sequence determines binding specificity to a target nucleotide. The heptameric sequence can be located anywhere within the  $\alpha$ -helical domain but it is preferred that the heptamer extend from position -1 to position 6 as the residues are conventionally numbered in the art. A peptide nucleotide-binding domain can include any  $\beta$ -sheet and framework sequences known in the art to function as part of a zinc finger protein.

10 Studies of natural zinc finger proteins have shown that three zinc finger domains can bind 9 bp of contiguous DNA sequence (Pavletich *et al.* (1991) *Science* 252:809-817; Swirnoff *et al.* (1995) *Mol. Cell. Biol.* 15:2275-2287). While recognition of 9 bp of sequence is insufficient to specify a unique site in a complex genome, proteins containing six zinc finger domains can specify 18-bp recognition (Liu *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:5525-5530). An 18-bp address made up of modular units is of sufficient complexity to specify a single site within all known genomes (see, published International PCT application No. WO 98/54311). Rules for constructing Zinc finger arrays that bind to a particular DNA sequence are known (see, *e.g.*, International PCT application No. WO 98/54311, which is based on U.S. application Serial No. 08/863,813; International PCT application No. 95/19431, which is based on U.S. application Serial Nos. 08/183,119 and 08/312,604).

25 Zinc finger-nucleotide binding polypeptide variants can be constructed from known motifs. The variants include at least two and preferably at least about four zinc finger modules that bind to a cellular

nucleotide sequence, such as DNA, RNA or both, and specifically bind to and modulate the function of a cellular nucleotide sequence.

For purposes herein, it is not necessary that the zinc finger-nucleotide binding motif be known in order to obtain a zinc-finger nucleotide binding variant polypeptide. It is contemplated that zinc finger-nucleotide binding motifs can be identified in non-eukaryotic DNA or RNA, especially in the native promoters of bacteria and viruses by the binding thereto of the modified nucleic acid binding peptides. Modified nucleic acid binding peptides should preserve the well known structural characteristics of the zinc finger, but differ from zinc finger proteins found in nature by their amino acid sequences and three-dimensional structures.

A variety of zinc finger proteins are known. Among these, the Cys<sub>2</sub>-His<sub>2</sub> (also referred to as "C2H2") zinc fingers are preferred for use in the fusion proteins. There are well-defined rules for C2H2 zinc finger binding to DNA that allow the DNA binding specificity of the fusion proteins containing the zinc fingers to be adjusted in order to reduce non-specific interactions with genes other than the targeted genes. These proteins can be selected or engineered to bind to diverse sequences. Further, the sequence specificity of these proteins can be modified to be different from their naturally occurring targets. Examples of zinc finger proteins from which a polypeptide can be produced include TFIIIA and Zif268.

The murine Cys<sub>2</sub>-His<sub>2</sub> zinc finger protein Zif268 has been used for construction of phage display libraries (Wu *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92:344-348). Zif268 is structurally the most well characterized of the zinc-finger proteins (Pavletich, *et al.* (1991) *Science* 252:809-817; Elrod-Erickson *et al.* (1996) *Structure* 4:1171-1180; Swirnoff *et al.* (1995) *Mol. Cell. Biol.* 15:2275-2287). DNA recognition in each of the three zinc finger domains of this protein is mediated by residues in the N-terminus of the  $\alpha$ -helix contacting primarily three nucleotides on a single strand of the DNA. The operator binding site for

this three finger protein is 5'-GCGIGGGCG-'3 (finger-2 subsite is underlined). Structural studies of Zif268 and other related zinc finger-DNA complexes have shown that residues from primarily three positions on the  $\alpha$ -helix, -1, 3, and 6, are involved in specific base contacts.

Typically, the residue at position -1 of the  $\alpha$ -helix contacts the 3' base of that finger's subsite while positions 3 and 6 contact the middle base and the 5' base, respectively.

**b. Construction and isolation of zinc finger DBD peptides**

A zinc finger-nucleotide binding polypeptide that binds to DNA, and specifically, the zinc finger domains that bind to DNA, can be identified by examination of the "linker" region between two zinc finger domains. The linker amino acid sequence TGEK(P) (SEQ ID NO: 19) is typically indicative of zinc finger domains that bind to a DNA. Therefore, one can determine whether a particular zinc finger-nucleotide binding polypeptide preferably binds to DNA or RNA by examination of the linker amino acids.

**c. Synthetic zinc fingers**

Synthetic zinc fingers can be assembled based upon known sequence specificities. A large number of zinc finger-nucleotide binding polypeptides were made and tested for binding specificity against target nucleotides containing a GNN triplet. The data show that a striking conservation of all three of the primary DNA contact positions (-1, 3, and 6) was observed for virtually all the clones of a given target (see, Example 1, see, also U.S. application Serial No. 09/173,941, filed 16 October, 1998, published as International PCT application No. WO 00/23464).

In order to select a family of zinc finger domains recognizing the 5'-GNN-3' subset of sequences, two highly diverse zinc finger libraries were constructed in the phage display vector pComb3H (Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. USA* 88:7978-7982; Rader *et al.* (1997) *Curr. Opin. Biotechnol.* 8:503-508). Both libraries involved randomization of residues within the  $\alpha$ -helix of finger 2 of C7, a variant of Zif268 (Wu *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92:344-348). Library 1 was

constructed by randomization of positions -1,1,2,3,5,6 using a NNK  
doping strategy while library 2 was constructed using a VNS doping  
strategy with randomization of positions -2,-1,1,2,3,5,6. The NNK  
doping strategy allows for all amino acid combinations within 32 codons  
while VNS precludes Tyr, Phe, Cys and all stop codons in its 24 codon  
set. The libraries contained  $4.4 \times 10^8$  and  $3.5 \times 10^9$  members,  
respectively, each capable of recognizing sequences of the 5'-  
GCGNNNGCG-3' type. The size of the NNK library ensured that it could  
be surveyed with 99% confidence while the VNS library was highly  
diverse but somewhat incomplete. These libraries are, however,  
significantly larger than previously reported zinc finger libraries  
(International PCT application No. WO 09/54311; Choo *et al.* (1994) *Proc  
Natl Acad Sci U S A* 91:11163-7; Greisman *et al.* (1997) *Science*  
275:657-661; Rebar *et al.* (1994) *Science* 263:671-673; Jamieson *et al.*  
(1994) *Biochemistry* 33:5689-5695; Jamieson *et al.* (1996) *Proc. Natl.  
Acad. Sci. U.S.A.* 93:12834-12839; Isalan *et al.* (1998) *Biochemistry*  
37:12026-12033; and U.S. Patent No. 5,789,538). Seven rounds of  
selection were performed on the zinc finger displaying-phage with each of  
the 16 5'-GCGNNNGCG-3' biotinylated hairpin DNAs targets using a  
solution binding protocol. Stringency was increased in each round by the  
addition of competitor DNA. Sheared herring sperm DNA was provided  
for selection against phage that bound non-specifically to DNA. Stringent  
selective pressure for sequence specificity was obtained by providing  
DNAs of the 5'-GCGNNNGCG-3' types as specific competitors. Excess  
DNA of the 5'-GCGNNNGCG-3' type was added to provide even more  
stringent selection against binding to DNAs with single or double base  
changes as compared to the biotinylated target. Phage binding to the  
single biotinylated DNA target sequence were recovered using  
streptavidin coated beads. In some cases the selection process was  
repeated. The data show that these domains are functionally modular  
and can be recombined with one another to create proteins capable of

binding to 18-bp sequences with subnanomolar affinity. The resulting family of zinc finger domains described herein is sufficient for the construction of 17 million proteins that bind to the 5'-(GNN)<sub>6</sub>-3' family of DNA sequences.

Also impressive amino acid conservation was been observed for recognition of the same nucleotide in different targets. For example, Asn in position 3 (Asn3) virtually always selects to recognize adenine in the middle position, whether in the context of GAG, GAA, GAT, or GAC. Gln-1 and Arg-1 were always selected to recognize adenine or guanine, respectively, in the 3' position regardless of context. Amide side chain based recognition of adenine by Gln or Asn is well documented in structural studies as is the Arg guanidinium side chain to guanine contact with a 3' or 5' guanine (see, *e.g.*, Elrod-Erickson *et al.* (1998) *Structure* 6:451-464).

More often, however, two or three amino acids are selected for nucleotide recognition. His3 or Lys3 (and to a lesser extent, Gly3) are selected for the recognition of a middle guanine. Ser3 and Ala3 are selected to recognize a middle thymine. Thr3, Asp3, and Glu3 are selected to recognize a middle cytosine. Asp and Glu were are selected in position -1 to recognize a 3' cytosine, while Thr-1 and Ser-1 are selected to recognize a 3' thymine.

Specific recognition of many nucleotides can best accomplished using motifs, rather than a single amino acid. For example, the best specification of a 3' guanine is achieved using the combination of Arg-1, Ser1, and Asp2 (the RSD motif). By using Val5 and Arg6 to specify a 5' guanine, recognition of subsites GGG, GAG, GTG, and GCG can be accomplished using a common helix structure (SRSD-X-LVR) differing only in the position 3 residue (Lys3 for GGG, Asn3 for GAG, Glu3 for GTG, and Asp3 for GCG). Similarly, 3' thymine is specified using Thr-1, Ser1, and Gly2 in the final clones(the TSG motif). Further, a 3' cytosine can be specified using Asp-1, Pro1, and Gly2 (the DPG motif) except when the

subsite is GCC; Pro1 is not tolerated by this subsite. Specification of a 3' adenine is with Gln-1, Ser1, Ser2 in two clones (QSS motif).

5 The data (see, Table 1 in Example) show that all possible GNN triplet sequences can be recognized with exquisite specificity by zinc finger domains. Optimized zinc finger domains can discriminate single base differences by greater than 100-fold loss in affinity. While many of the amino acids found in the optimized proteins at the key contact positions -1,3, and 6 are those that are consistent with a simple code of recognition, it has been discovered that optimal specific recognition is sensitive to the context in which these residues are presented. Residues at positions 1,2, and 5 have been found to be critical for specific recognition.

10 Further the data demonstrate that sequence motifs at positions -1,1, and 2 rather than the simple identity of the position 1 residue are required for highly specific recognition of the 3' base. These residues likely provide the proper stereo-chemical context for interactions of the helix in terms of recognition of specific bases and in the exclusion of other bases, the net result being highly specific interactions. Ready recombination of the disclosed domains then allows for the creation of proteins, typically polypdactyl proteins, of defined specificity precluding the need to develop phage display libraries in their generation. Such family of zinc finger domains is sufficient for the construction of 16 or 17 million proteins that bind to the 5'-(GNN)<sub>6</sub>-3' family of DNA sequences.

#### 15 d. Modification of zinc finger peptides

20 The zinc finger-nucleotide binding peptide domain can be derived or produced from a wild type zinc finger protein by truncation or expansion, or as a variant of the wild type-derived polypeptide by a process of site directed mutagenesis, or by a combination of the procedures (see, *e.g.*, U.S. Patent No. 5,789,538, which describes methods for design and construction of zinc finger peptides). Mutagenesis can be performed to replace non-conserved residues in one or more of the repeats of the

consensus sequence. Truncated zinc finger-nucleotide binding proteins can also be mutagenized.

DNA encoding the zinc finger-nucleotide binding proteins, including native, truncated, and expanded polypeptides, can be obtained by several methods. For example, the DNA can be isolated using hybridization procedures which are well known in the art. These include, but are not limited to: (1) hybridization of probes to genomic or cDNA libraries to detect shared nucleotide sequences; (2) antibody screening of expression libraries to detect shared structural features; and (3) synthesis by the polymerase chain reaction (PCR). RNA can be obtained by methods known in the art (see *e.g.*, *Current Protocols in Molecular Biology*, 1988, Ed. Ausubel, *et al.*, Greene Publish. Assoc. & Wiley Interscience).

DNA encoding zinc finger-nucleotide binding proteins also can be obtained by: (1) isolation of a double-stranded DNA sequence from the genomic DNA; (2) chemical manufacture of a DNA sequence to provide the necessary codons for the polypeptide of interest; and (3) *in vitro* synthesis of a double-stranded DNA sequence by reverse transcription of mRNA isolated from a eukaryotic donor cell. In the latter case, a double-stranded DNA complement of mRNA is eventually formed which is generally referred to as cDNA. Of these three methods the isolation of genomic DNA is the least common. This is especially true when it is desirable to obtain the microbial expression of mammalian polypeptides due to the presence of introns.

For obtaining zinc finger derived-DNA binding polypeptides, the synthesis of DNA sequences is frequently the method of choice when the entire sequence of amino acid residues of the desired polypeptide product is known. When the entire sequence of amino acid residues of the desired polypeptide is not known, the direct synthesis of DNA sequences is not possible and the method of choice is the formation of cDNA sequences. Among the standard procedures for isolating cDNA sequences of interest is the formation of plasmid-carrying cDNA libraries

which are derived from reverse transcription of mRNA which is abundant in donor cells that have a high level of genetic expression. When used in combination with polymerase chain reaction technology, even rare expression products can be cloned. In those cases where significant portions of the amino acid sequence of the polypeptide are known, the production of labeled single or double-stranded DNA or RNA probe sequences duplicating a sequence putatively present in the target cDNA may be employed in DNA/DNA hybridization procedures which are carried out on cloned copies of the cDNA which have been denatured into a single-stranded form (Jay, *et al.*, *Nucleic Acid Research*, 11:2325, 1983).

Hybridization procedures are useful for the screening of recombinant clones by using labeled mixed synthetic oligonucleotide probes where each probe is potentially the complete complement of a specific DNA sequence in the hybridization sample which includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA. Hybridization is particularly useful in the detection of cDNA clones derived from sources where an extremely low amount of mRNA sequences relating to the polypeptide of interest are present. By using stringent hybridization conditions directed to avoid non-specific binding, it is possible, for example, to allow the autoradiographic visualization of a specific cDNA clone by the hybridization of the target DNA to that single probe in the mixture which is its complete complement (Wallace, *et al.*, *Nucleic Acid Research*, 9:879, 1981; Maniatis, *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, 1982).

Screening procedures that rely on nucleic acid hybridization make it possible to isolate any gene sequence from any organism, provided the appropriate probe is available. Oligonucleotide probes, which correspond to a part of the sequence encoding the protein in question, can be synthesized chemically. This requires that short, oligopeptide stretches of

amino acid sequence must be known. The DNA sequence encoding the protein can be deduced from the genetic code, however, the degeneracy of the code must be taken into account. It is possible to perform a mixed addition reaction when the sequence is degenerate. This includes a heterogeneous mixture of denatured double-stranded DNA. For such screening, hybridization is preferably performed on either single-stranded DNA or denatured double-stranded DNA.

A cDNA expression library, such as lambda gt11, can be screened indirectly for zinc finger-nucleotide binding protein or for the zinc finger derived polypeptide having at least one epitope, using antibodies specific for the zinc finger-nucleotide binding protein. Such antibodies can be either polyclonally or monoclonally derived and used to detect expression product indicative of the presence of zinc finger-nucleotide binding protein cDNA. Alternatively, binding of the derived polypeptides to DNA targets can be assayed by incorporated radiolabeled DNA into the target site and testing for retardation of electrophoretic mobility as compared with unbound target site.

A preferred vector used for identification of truncated and/or mutagenized zinc finger-nucleotide binding polypeptides is a recombinant DNA molecule containing a nucleotide sequence that codes for and is capable of expressing a fusion polypeptide containing, in the direction of amino- to carboxy-terminus, (1) a prokaryotic secretion signal domain, (2) a heterologous polypeptide, and (3) a filamentous phage membrane anchor domain. The vector includes DNA expression control sequences for expressing the fusion polypeptide, preferably prokaryotic control sequences.

Since the DNA sequences provided herein encode essentially all or part of a zinc finger-nucleotide binding protein, it is routine to prepare, subclone, and express the truncated polypeptide fragments of DNA from this or corresponding DNA sequences. Alternatively, by using the DNA fragments disclosed herein, which define the zinc finger-nucleotide

binding polypeptides, it is possible, in conjunction with known techniques, to determine the DNA sequences encoding the entire zinc finger-nucleotide binding protein. Such techniques are described in U.S. 4,394,443 and U.S. 4,446,235, which are incorporated herein by reference.

In addition to modifications in the amino acids making up the zinc finger, the zinc finger derived polypeptide can contain more or less than the full amount of fingers contained in the wild type protein from which it is derived. Minor modifications of the primary amino acid sequence may result in proteins which have substantially equivalent activity compared to the zinc finger derived-binding protein described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous. All proteins produced by these modifications are included herein as long as zinc finger-nucleotide binding protein activity exists.

**e. Screening of variant zinc finger and other DBD peptides**

Any method known to those of skill in the art for identification of functional modular domains derived from zinc fingers and combinations thereof can be employed. An exemplary method for identifying variants of zinc fingers or other polypeptides that bind to zinc finger binding motifs is provided. Components used in the method include a nucleic acid molecule encoding a putative or modified zinc finger peptide operably linked to a first inducible promoter and a reporter gene operably linked to a second inducible promoter and a zinc finger-nucleotide binding motif, wherein the incubating is carried out under conditions sufficient to allow the components to interact, and measuring the affect of the putative DBD peptide on the expression of the reporter gene is provided.

For example, a first inducible promoter, such as the arabinose promoter, is operably linked to the nucleotide sequence encoding the putative DBD polypeptide. A second inducible promoter, such as the

lactose promoter, is operably linked to a zinc finger derived-DNA binding motif followed by a reporter gene, such as  $\beta$ -galactosidase. Incubation of the components may be *in vitro* or *in vivo*. *In vivo* incubation may include prokaryotic or eukaryotic systems, such as *E.coli* or COS cells, respectively. Conditions that allow the assay to proceed include incubation in the presence of a substance, such as arabinose and lactose, which activate the first and second inducible promoters, respectively, thereby allowing expression of the nucleotide sequence encoding the putative trans-modulating protein nucleotide sequence. Determination of whether the putative modulating protein binds to the zinc finger-nucleotide binding motif, which is operably linked to the second inducible promoter, and affects its activity is measured by the expression of the reporter gene. For example, if the reporter gene is  $\beta$ -galactosidase, the presence of blue or white plaques indicates whether the putative modulating protein enhances or inhibits, respectively, gene expression from the promoter. Other commonly used assays to assess the function from a promoter, including chloramphenicol acetyl transferase (CAT) assay, are known to those of skill in the art. Prokaryote and eukaryote systems can be used.

As discussed above, Example 1 provides an illustration of modification of Zif268 as described above. Therefore, in another embodiment, a ligand activated transcriptional regulator polypeptide variant containing at least two zinc finger modules that bind to an HIV sequence and modulates the function of the HIV sequence, for example, the HIV promoter sequence is provided.

In another embodiment, zinc finger proteins can be manipulated to recognize and bind to extended target sequences. For example, zinc finger proteins containing from about 2 to 20 zinc fingers Zif(2) to Zif(20), and preferably from about 2 to 12 zinc fingers, may be fused to the leucine zipper domains of the Jun/Fos proteins, prototypical members of the bZIP family of proteins (O'Shea *et al.* (1991) *Science* 254:539).

Alternatively, zinc finger proteins can be fused to other proteins which are capable of forming heterodimers and contain dimerization domains. Such proteins are known to those of skill in the art.

The Jun/Fos leucine zippers are described for illustrative purposes and preferentially form heterodimers and allow for the recognition of 12 to 72 base pairs. Henceforth, Jun/Fos refer to the leucine zipper domains of these proteins. Zinc finger proteins are fused to Jun, and independently to Fos by methods commonly used in the art to link proteins. Following purification, the Zif-Jun and Zif-Fos constructs, the proteins are mixed to spontaneously form a Zif-Jun/Zif-Fos heterodimer. Alternatively, coexpression of the genes encoding these proteins results in the formation of Zif-Jun/Zif-Fos heterodimers *in vivo*. Fusion of the heterodimer with an N-terminal nuclear localization signal allows for targeting of expression to the nucleus (Calderon, *et al*, *Cell*, 41:499, 1982). Activation domains may also be incorporated into one or each of the leucine zipper fusion constructs to produce activators of transcription (Sadowski *et al*. (1992) *Gene* 118:137). These dimeric constructs then allow for specific activation or repression of transcription. These heterodimeric Zif constructs are advantageous since they allow for recognition of palindromic sequences (if the fingers on Jun and Fos recognize the same DNA/RNA sequence) or extended asymmetric sequences (if the fingers on Jun and Fos recognize different DNA/RNA sequences). For example the palindromic sequence

5' - GGC CCA CGC {N}<sub>x</sub> GCG TGG GCG - 3'

3' - GCG GGT GCG {N}<sub>x</sub> CGC ACC CGC - 5' (SEQ ID NO: 20)

is recognized by the Zif268-Fos/Zif268 Jun dimer (x is any number). The spacing between subsites is determined by the site of fusion of Zif with the Jun or Fos zipper domains and the length of the linker between the Zif and zipper domains. Subsite spacing is determined by a binding site selection method as is common to those skilled in the art (Thiesen *et al*. (1990) *Nucleic Acids Research*, 18:3203, 1990). Example of the

recognition of an extended asymmetric sequence is shown by the Zif(C7)<sub>6</sub>-Jun/Zif-268-Fos dimer. This protein includes 6 fingers of the C7 type (EXAMPLE 11) linked to Jun and three fingers of Zif268 linked to Fos, and recognizes the extended sequence:

5' - CGC CGC CGC CGC CGC CGC {N}<sub>x</sub> GCG TGG GCG - 3'  
 3' - GCG GCG GCG GCG GCG GCG {N}<sub>x</sub> CGC ACC CGC - 5'  
 (SEQ ID NO: 21)

In another embodiment, attachment of chelating groups to Zif proteins is preferably facilitated by the incorporation of a Cysteine (Cys) residue between the initial Methionine (Met) and the first Tyrosine (Tyr) of the protein. The Cys is then alkylated with chelators known to those skilled in the art, for example, EDTA derivatives as described (Sigman (1990) *Biochemistry*, 29:9097). Alternatively the sequence Gly-Gly-His can be made as the most amino terminal residues since an amino terminus composed of the residues has been described to chelate Cu<sup>+2</sup> (Mack *et al.* (1988) *J. Am. Chem. Soc.* 110:7572). Preferred metal ions include Cu<sup>+2</sup>, Ce<sup>+3</sup> (Takasaki and Chin (1994) *J. Am. Chem. Soc.* 116:1121, 1994) Zn<sup>+2</sup>, Cd<sup>+2</sup>, Pb<sup>+2</sup>, Fe<sup>+2</sup> (Schnaith *et al.* (1994) *Proc. Natl. Acad. Sci., USA* 91:569, 1994), Fe<sup>+3</sup>, Ni<sup>+2</sup>, Ni<sup>+3</sup>, La<sup>+3</sup>, Eu<sup>+3</sup> (Hallet *al.* (1994) *Chemistry and Biology* 1:185), Gd<sup>+3</sup>, Tb<sup>+3</sup>, Lu<sup>+3</sup>, Mn<sup>+2</sup>, Mg<sup>+2</sup>. Cleavage with chelated metals is generally performed in the presence of oxidizing agents such as O<sub>2</sub>, hydrogen peroxide H<sub>2</sub>O<sub>2</sub> and reducing agents such as thiols and ascorbate. The site and strand (+ or - site) of cleavage is determined empirically (Mack *et al.* (1988) *J. Am. Chem. Soc.* 110:7572, 1988) and is dependent on the position of the Cys between the Met and the Tyr preceding the first finger. In the protein Met (AA) Tyr-(Zif)<sub>1-12</sub>, the chelate becomes Met-(AA)<sub>x1</sub> Cys-Chelate-(AA)<sub>x2</sub>-Tyr-(Zif)<sub>1-12</sub>, where AA = any amino acid and x = the number of amino acids. Dimeric zif constructs of the type Zif-Jun/Zif-Fos are preferred for cleavage at two sites within the target oligonucleotide or at a single long target site. In the case where double stranded cleavage is desired, Jun

and Fos containing proteins are labelled with chelators and cleavage is performed by methods known to those skilled in the art. In this case, a staggered double-stranded cut analogous to that produced by restriction enzymes is generated.

Following mutagenesis and selection of variants of the Zif268 protein in which the finger 1 specificity or affinity is modified, proteins carrying multiple copies of the finger may be constructed using the TGEKP linker sequence by methods known in the art. For example, the C7 finger may be constructed according to the scheme:

MKLLPEYACPVESCDRRFSKSADLKRHIRHTTGEKP- (SEQ ID NO: 22) (YACPVESCDRRFSKSADLKHIRIHTTGEKP)<sub>1-11</sub>, (SEQ ID NO: 23) where the sequence of the last linker is subject to change since it is at the terminus and not involved in linking two fingers together. This protein binds the designed target sequence GCG-GCG-GCG in the oligonucleotide hairpin CCT-CGC-CGC-CGC-GGG-TTT-TCC-CGC-GCC-CCC GAG G (SEQ ID NO: 24) with an affinity of 9nM, as compared to an affinity of 300 nM for an oligonucleotide encoding the GCG-TGG-GCG sequence (as determined by surface plasmon resonance studies). Fingers used need not be identical and may be mixed and matched to produce proteins which recognize a desired target sequence. These may also be used with leucine zippers (*e.g.*, Fos/Jun) or other heterodimers to produce proteins with extended sequence recognition.

In addition to producing polymers of finger 1, the entire three finger Zif268 and modified versions therein may be fused using the consensus linker TGEKP to produce proteins with extended recognition sites. For example, the protein Zif268-Zif268 can be produced in which the natural protein has been fused to itself using the TGEKP linker. This protein now binds the sequence GCG-TGG-GCG-GCG-TGG-GCG. Therefore modifications within the three fingers of Zif268 or other zinc finger proteins known in the art may be fused together to form a protein which

recognizes extended sequences. These new zinc proteins may also be used in combination with leucine zippers if desired.

### 3. Transcription regulating domain (TRD)

Any TRD known to those of skill in the art can be selected, including those present in intracellular receptors. The TRD is selected to regulate transcription of the gene targeted by the DBD and to effect regulation of expression thereof. The TRD can be selected to regulate expression of an endogenous gene in a cell or in an exogenously added construct. For exogenously added genes, the regulatory region of the gene can be selected to interact with a desired TRD. Identification, preparation and testing of TRDs in combination with DBDs is exemplified herein for ERB-2 and integrin  $\beta_3$ .

#### a. Selection of the TRD

Transcription regulating domains are well known in the art. Exemplary and preferred transcription repressor domains are ERD, KRAB, SID, Deacetylase, and derivatives, multimers and combinations thereof such as KRAB-ERD, SID-ERD, (KRAB)<sub>2</sub>, (KRAB)<sub>3</sub>, KRAB-A, (KRAB-A)<sub>2</sub>, (SID)<sub>2</sub> (KRAB-A)-SID and SID-(KRAB-A).

#### b. Repressors

Transcriptional repressors are well known in the art, and any such repressor can be used herein. The repressor is a polypeptide that is operatively linked to the nucleic acid binding domain as set forth above. The repressor is operatively linked to the binding domain in that it is attached to the binding domain in such a manner that, when bound to a target nucleotide via that binding domain, the repressor acts to inhibit or prevent transcription. The repressor domain can be linked to the binding domain using any linking procedure well known in the art. It may be necessary to include a linker moiety between the two domains. Such a linker moiety is typically a short sequence of amino acid residues that provides spacing between the domains. So long as the linker does not

interfere with any of the functions of the binding or repressor domains, any sequence can be used.

Transcriptional repressors have been generated by attaching either of three human-derived repressor domains to the zinc finger protein. The first repressor protein was prepared using the ERF repressor domain (ERD) (Sgouras *et al.* (1995) *EMBO J.* 14:4781-4793), defined by amino acids 473 to 530 of the *ets2* repressor factor (ERF). This domain mediates the antagonistic effect of ERF on the activity of transcription factors of the *ets* family. A synthetic repressor was constructed by fusion of this domain to the C-terminus of the zinc finger protein.

The second repressor protein was prepared using the Krüppel-associated box (KRAB) domain (Margolin *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:4509-4513). This repressor domain is commonly found at the N-terminus of zinc finger proteins and presumably exerts its repressive activity on TATA-dependent transcription in a distance- and orientation-independent manner (Pengue *et al.* (1996) *Proc. Natl. Acad. Sci. USA* 93:1015-1020), by interacting with the RING finger protein KAP-1 (Friedman *et al.* (1996) *Genes & Dev.* 10:2067-2078). The KRAB domain found between amino acids 1 and 97 of the zinc finger protein KOX1 (Margolin *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:4509-4513) was used. In this case an N-terminal fusion with the six-finger protein was constructed.

Histone deacetylation as a means for repression can be employed. For example, amino acids 1 to 36 of the Mad mSIN3 interaction domain (SID) have been fused to the N-terminus of a zinc finger protein (Ayer *et al.* (1996) *Mol. Cell. Biol.* 16:5772-5781). This small domain is found at the N-terminus of the transcription factor Mad and is responsible for mediating its transcriptional repression by interacting with mSIN3, which in turn interacts the co-repressor N-CoR and with the histone deacetylase mRPD1 (Heinzel *et al.* (1997) *Nature* 387:43-46).

### c. Activators

Exemplary and preferred transcription activation domains include any protein or factor that regulates transcription. Exemplary transcriptional regulation domains include, but are not limited to, VP16, TA2, VP64, STAT6 and relA.

#### 4. Exemplary construct based on human integrin $\beta 3$ and erbB-2 target sequences

To exemplify the generation of zinc finger modular domains and peptides containing one or more of such domains to produce peptides with DNA binding specificity and therapeutic potential, target sequences have been identified based on human integrin  $\beta 3$  and *erbB-2* (Ishii *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:4374-4378) genomic sequences.

##### *Integrin $\beta 3$ as a target for cancer gene therapy*

Integrin  $\alpha_v\beta_3$  is the most promiscuous member of the integrin family and has been identified as a marker of angiogenic vascular tissue. For instance, integrin  $\alpha_v\beta_3$  shows enhanced expression on blood vessels in human wound granulation tissue but not in normal skin. Following the induction of angiogenesis, blood vessels show a four-fold increase in  $\alpha_v\beta_3$  expression compared to blood vessels not undergoing this process. It has been reported that a cyclic peptide or monoclonal antibody antagonist of integrin  $\alpha_v\beta_3$  blocks cytokine- or tumor-induced angiogenesis on the chick chorioallantoic membrane. Therefore, inhibition of integrin  $\alpha_v\beta_3$  expression provides an approach to block tumor-induced angiogenesis.

##### *ErbB-2 receptor tyrosine kinases as a target for cancer gene therapy*

Members of the ErbB receptor family play an important role in the development of human malignancies. In particular, ErbB-2 is over-expressed as a result of gene amplification and/or transcriptional deregulation in a high percentage of human adenocarcinomas arising at numerous sites, including breast, ovary, lung, stomach, and salivary gland. Increased expression of ErbB-2 *per se* leads to constitutive activation of its intrinsic tyrosine kinase. Many clinical studies have

shown that patients with tumors showing elevated expression of ErbB-2 have poorer prognosis. Thus, the high occurrence of its aberrant expression in human cancer, as well as the aggressive behavior of over-expressing tumors, make ErbB-2 an attractive target for therapy.

5           Generation and construction of zinc fingers and fusion proteins targeted to *erbB-2* and integrin  $\beta_3$  are described in the EXAMPLES.

#### **B.     Regulatable cassette**

10           In embodiments in which the targeted gene is an exogenous gene, particularly a gene that encodes a therapeutic product, the gene is provided as in an expression cassette operatively linked to a promoter and regulatory region with which the fusion protein specifically interacts.

The cassette includes at least one polynucleotide domain recognized by the corresponding zinc finger domain present in the fusion protein and a suitable promoter to direct transcription of the exogenous gene.

15           Typically, the regulatable expression cassette contains three to six response elements and interacts with nucleic acid binding domain of the ligand activated transcriptional regulatory fusion protein.

20           Typically the exogenous gene encodes a therapeutic product, such as a growth factor, that can supplement peptides, polypeptides or proteins encoded by endogenous expressed genes, thereby providing an effective therapy. In several embodiments the gene encodes a suitable reporter molecule that can be detected by suitable direct or indirect means. The cassette can be inserted into a suitable delivery vehicle for introduction into cells. Such vehicles include, but are not limited to, 25 human adenovirus vectors, adeno-associated vectors, murine or lenti virus derived retroviral vectors, and a variety of non-viral compositions including liposomes, polymers, and other DNA containing conjugates.

#### **C.     Use of the fusion proteins for gene regulation**

##### **1.     Delivery of the nucleic acids**

30           There are available to one skilled in the art multiple viral and non-viral methods suitable for introduction of a nucleic acid molecule into a

target cell. Genetic modification of a cell may be accomplished using one or more techniques well known in the gene therapy field (Human Gene Therapy, April 1994, Vol. 5, p. 543-563; Mulligan, R.C. 1993).

5 The ability to regulate transgene expression, as defined in the examples herein, can be applied to a wide variety of applications for gene therapy. The ability to control expression of an exogenously introduced transgene is important for the safety and efficacy of most or all envisioned cell and gene therapies. Control of transgene expression can be used to accomplish regulation of a therapeutic protein level, ablation of  
10 a desired cell population, either the vector containing cells or others, or activation of a recombinase or other function resulting in control of vector function within the transduced cells. Further, such control permits termination of a gene therapy treatment if necessary.

15 A number of vector systems useful for gene therapy have been described previously in this application. Vectors for gene therapy include any known to those of skill in the art, and include any vectors derived from animal viruses and artificial chromosomes. The vectors may be designed for integration into the host cell's chromosomes or to remain as extrachromosomal elements. Such vectors include, but are not limited to human adenovirus vectors, adeno-associated viral vectors, retroviral  
20 vectors, such as murine retroviral vectors and lentivirus-derived retroviral vectors. Also contemplated herein are any of the variety of non-viral compositions for targeting and/or delivery of genetic material, including, but are not limited to, liposomes, polymers, and other DNA containing compositions, and targeted conjugates, such as nucleic acids linked to  
25 antibodies and growth factors. Any delivery system is intended for use of delivery of the nucleic acid constructs encoding the fusion polypeptide and also targeted exogenous genes. Such vector systems can be used to deliver the ZFP-LBD fusion proteins and the inducible transgene cassette either in vitro or in vivo, depending on the vector system. With  
30 adenovirus, for instance, vectors can be administered intravenously to

transduce the liver and other organs, introduced directly into the lung, or into vascular compartments temporarily localized by ligation or other methods. Methods for constructing such vectors, and methods and uses thereof are known to those skilled in the field of gene therapy.

5 In one embodiment, one vector encodes the fusion protein regulator and a second vector encodes the inducible transgene cassette. Vectors can be mixed or delivered sequentially to incorporate into cells the regulator and transgene at the appropriate amounts. Subsequent administration of an effective amount of the ligand by standard routes would result in activation of the transgene.

10 In another embodiment, the nucleic acid encoding the fusion protein and the inducible transgene can be included in the same vector construction. In this instance, the nucleic acid encoding the fusion protein would be positioned within the vector and expressed from a promoter in such a way that it did not interfere with the basal expression and inducibility of the transgene cassette. Further, the use of cell or tissue specific promoters to express the fusion protein confers an additional level of specificity on the system. Dual component vectors and use for gene therapy are known (see, *e.g.*, Burcin *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96: 335-360, which describes an adenovirus vector fully deleted of viral backbone genes).

15 In another embodiment, gene therapy can be accomplished using a combination of the vectors described above. For example, a retroviral vector can deliver a stably integrated, inducible transgene cassette into a population of cells either *in vitro* (*ex vivo*) or *in vivo*. Subsequently, the integrated transgene can be activated by transducing this same cell population with a second vector, such as an adenovirus vector capable of expressing the fusion protein, followed by the administration of the specific ligand inducing agent. This is particularly useful where "one time" activation of the transgene is desired, for example as a cellular suicide mechanism. An example of this application is the stable

integration of an inducible transgene cassette containing the herpes simplex virus thymidine kinase gene (HSV Tk). Subsequent activation of this gene confers sensitivity to ganciclovir and allows ablation of this modified cell.

**a. Viral Delivery systems**

Viral transduction methods for delivering nucleic acid constructs to cells are contemplated herein. Suitable DNA viral vectors for use herein includes, but are not limited to an adenovirus (Ad), adeno-associated virus (AAV), herpes virus, vaccinia virus or a polio virus. A suitable RNA virus for use herein includes but is not limited to a retrovirus or Sindbis virus. It is to be understood by those skilled in the art that several such DNA and RNA viruses exist that may be suitable for use herein. Adenoviral vectors have proven especially useful for gene transfer into eukaryotic cells and are widely available to one skilled in the art and is suitable for use herein.

Adeno-associated virus (AAV) has recently been introduced as a gene transfer system with potential applications in gene therapy. Wild-type AAV demonstrates high-level infectivity, broad host range and specificity in integrating into the host cell genome. Herpes simplex virus type-1 (HSV-1) vectors are available and are especially useful in the nervous system because of its neurotropic property. Vaccinia viruses, of the poxvirus family, have also been developed as expression vectors. Each of the above-described vectors is widely available and is suitable for use herein.

Retroviral vectors are capable of infecting a large percentage of the target cells and integrating into the cell genome. Preferred retroviruses include lentiviruses, such as but are not limited to, HIV, BIV and SIV.

Various viral vectors that can be used for gene therapy as taught herein include adenovirus, herpes virus, vaccinia, adeno-associated virus (AAV), or, preferably, an RNA virus such as a retrovirus. Preferably, the retroviral vector is a derivative of a murine or avian retrovirus, or is a

lentiviral vector. The preferred retroviral vector is a lentiviral vector. Examples of retroviral vectors in which a single foreign gene can be inserted include, but are not limited to: Moloney murine leukemia virus (MoMuLV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), SIV, BIV, HIV and Rous Sarcoma Virus (RSV). A number of additional retroviral vectors can incorporate multiple genes. All of these vectors can transfer or incorporate a gene for a selectable marker so that transduced cells can be identified and generated. By inserting a zinc finger derived-DNA binding polypeptide sequence of interest into the viral vector, along with another gene that encodes the ligand for a receptor on a specific target cell, for example, the vector is made target specific. Retroviral vectors can be made target specific by inserting, for example, a polynucleotide encoding a protein. Preferred targeting is accomplished by using an antibody to target the retroviral vector. Those of skill in the art know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the retroviral genome to allow target specific delivery of the retroviral vector containing the zinc finger-nucleotide binding protein polynucleotide.

Since recombinant retroviruses are defective, they require assistance in order to produce infectious vector particles. This assistance can be provided, for example, by using helper cell lines that contain plasmids encoding all of the structural genes of the retrovirus under the control of regulatory sequences within the LTR. These plasmids are missing a nucleotide sequence which enables the packaging mechanism to recognize an RNA transcript for encapsitation. Helper cell lines which have deletions of the packaging signal include but are not limited to  $\Psi 2$ , PA317 and PA12, for example. These cell lines produce empty virions, since no genome is packaged. If a retroviral vector is introduced into such cells in which the packaging signal is intact, but the structural genes are replaced by other genes of interest, the vector can be packaged and

vector virion produced. The vector virions produced by this method can then be used to infect a tissue cell line, such as NIH 3T3 cells, to produce large quantities of chimeric retroviral virions.

**b. Nonviral Delivery systems**

"Non-viral" delivery techniques for gene therapy include DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA,  $\text{CaPO}_4$  precipitation, gene gun techniques, electroporation, liposomes and lipofection. Any of these methods are available to one skilled in the art and would be suitable for use herein. Other suitable methods are available to one skilled in the art, and it is to be understood that the herein may be accomplished using any of the available methods of transfection.

Another targeted delivery system is a colloidal dispersion system. Colloidal dispersion systems include macromolecule complexes, nanocapsules, microspheres, beads, and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes, which are preferred. Liposomes are artificial membrane vesicles which are useful as delivery vehicles *in vitro* and *in vivo*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2-4.0  $\mu\text{m}$  can encapsulate a substantial percentage of an aqueous buffer containing large macromolecules. RNA, DNA and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraleigh, *et al.*, *Trends Biochem. Sci.*, 6:77, 1981).

Lipofection may be accomplished by encapsulating an isolated nucleic acid molecule within a liposomal particle and contacting the liposomal particle with the cell membrane of the target cell. Liposomes are self-assembling, colloidal particles in which a lipid bilayer, composed of amphiphilic molecules such as phosphatidyl serine or phosphatidyl choline, encapsulates a portion of the surrounding media such that the lipid bilayer surrounds a hydrophilic interior. Unilamellar or multilamellar liposomes can be constructed such that the interior

contains a desired chemical, drug, or, as provide herein, an isolated nucleic acid molecule.

Liposomes have been used for delivery of polynucleotides in plant, yeast and bacterial cells as well as mammalian cells. In order for a liposome to be an efficient gene transfer vehicle, characteristics among the following should be present: (1) encapsulation of the genes of interest at high efficiency while not compromising their biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information (Mannino, *et al.*, *Biotechniques*, 6:682, 1988).

The composition of the liposome is usually a combination of phospholipids, particularly high-phase-transition-temperature phospholipids, usually in combination with steroids, especially cholesterol. Other phospholipids or other lipids may also be used. The physical characteristics of liposomes depend on pH, ionic strength, and the presence of divalent cations.

Examples of lipids useful in liposome production include phosphatidyl compounds, such as phosphatidylglycerol, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, sphingolipids, cerebroside, and gangliosides. Particularly useful are diacylphosphatidylglycerols, where the lipid moiety contains from 14-18 carbon atoms, particularly from 16-18 carbon atoms, and is saturated. Illustrative phospholipids include egg phosphatidylcholine, dipalmitoylphosphatidylcholine and distearoylphosphatidylcholine.

The targeting of liposomes has been classified based on anatomical and mechanistic factors. Anatomical classification is based on the level of selectivity, for example, organ-specific, cell-specific, and organelle-specific. Mechanistic targeting can be distinguished based upon whether it is passive or active. Passive targeting uses the natural tendency of

liposomes to distribute to cells of the reticulo-endothelial system (RES) in organs which contain sinusoidal capillaries. Active targeting, on the other hand, involves alteration of the liposome by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein, or by changing the composition or size of the liposome in order to achieve targeting to organs and cell types other than the naturally occurring sites of localization.

The surface of the targeted delivery system may be modified in a variety of ways. In the case of a liposomal targeted delivery system, lipid groups can be incorporated into the lipid bilayer of the liposome in order to maintain the targeting ligand in stable association with the liposomal bilayer. Various linking groups can be used for joining the lipid chains to the targeting ligand.

In general, the compounds bound to the surface of the targeted delivery system are ligands and receptors permitting the targeted delivery system to find and "home in" on the desired cells. A ligand may be any compound of interest that interacts with another compound, such as a receptor.

In general, surface membrane proteins that bind to specific effector molecules are referred to as receptors. Antibodies are preferred receptors. Antibodies can be used to target liposomes to specific cell-surface ligands. For example, certain antigens expressed specifically on tumor cells, referred to as tumor-associated antigens (TAAs), may be exploited for the purpose of targeting antibody-zinc finger-nucleotide binding protein-containing liposomes directly to the malignant tumor. Since the zinc finger-nucleotide binding protein gene product may be indiscriminate with respect to cell type in its action, a targeted delivery system offers a significant improvement over randomly injecting non-specific liposomes. A number of procedures can be used to covalently attach either polyclonal or monoclonal antibodies to a liposome bilayer. Antibody-targeted liposomes can include monoclonal or polyclonal

antibodies or fragments thereof such as Fab, or F(ab')<sub>2</sub>, as long as they bind efficiently to an the antigenic epitope on the target cells. Liposomes may also be targeted to cells expressing receptors for hormones or other serum factors.

## 2. Administration

### a. Delivery of constructs to cells

The cells may be transfected *in vivo*, *ex vivo* or *in vitro*. The cells may be transfected as primary cells isolated from a patient or a cell line derived from primary cells, and are not necessarily autologous to the patient to whom the cells are ultimately administered. Following *ex vivo* or *in vitro* transfection, the cells may be implanted into a host. Genetic modification of the cells may be accomplished using one or more techniques well known in the gene therapy field (see, *e.g.*, (1994) *Human Gene Therapy* 5:543-563).

Administration of a nucleic acid molecules provided herein to a target cell *in vivo* may be accomplished using any of a variety of techniques well known to those skilled in the art. The vectors of the herein may be administered orally, parentally, by inhalation spray, rectally, or topically in dosage unit formulations containing conventional pharmaceutically acceptable carriers, adjuvants, and vehicles. Suppositories for rectal administration of the drug can be prepared by mixing the drug with a suitable non-irritating excipient such as cocoa butter and polyethylene glycols that are solid at ordinary temperatures but liquid at the rectal temperature and therefore melt in the rectum and release the drug.

The dosage regimen for treating a disorder or a disease with the vectors and/or compositions provided is based on a variety of factors, including the type of disease, the age, weight, sex, medical condition of the patient, the severity of the condition, the route of administration, and the particular compound employed. Thus, the dosage regimen may vary widely, but can be determined empirically using standard methods.

The pharmaceutically active compounds (i.e., vectors) can be processed in accordance with conventional methods of pharmacy to produce medicinal agents for administration to patients, including humans and other mammals. For oral administration, the pharmaceutical composition may be in the form of, for example, a capsule, a tablet, a suspension, or liquid. The pharmaceutical composition is preferably made in the form of a dosage unit containing a given amount of DNA or viral vector particles (collectively referred to as "vector"). For example, these may contain an amount of vector from about  $10^3$ - $10^{15}$  viral vector particles, preferably from about  $10^6$ - $10^{12}$  viral particles. A suitable daily dose for a human or other mammal may vary widely depending on the condition of the patient and other factors, but, once again, can be determined using routine methods. The vector may also be administered by injection as a composition with suitable carriers including saline, dextrose, or water.

While the nucleic acids and /or vectors herein can be administered as the sole active pharmaceutical agent, they can also be used in combination with one or more vectors or other agents. When administered as a combination, the therapeutic agents can be formulated as separate compositions that are given at the same time or different times, or the therapeutic agents can be given as a single composition.

#### **b. Deliver ligand**

Ligands similarly may be delivered by any suitable mode of administration, including by oral, parenteral, intravenous, intramuscular and other known routes. Any known pharmaceutical formulations is contemplated.

### **3. Ligands**

As noted, the ligands may be naturally-occurring ligands, but are preferentially non-natural ligands with which the LBD is modified to specifically interact. Methods for modifying the LBD are known, as are methods for screening for such ligands.

Ligands include, non-natural ligands, hormones, anti-hormones, synthetic hormones, and other such compounds. Examples of non-natural ligands, anti-hormones and non-native ligands include, but are not limited to, the following: 11 $\beta$ -4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\alpha$ -propinyl-4,9-e stradiene-3-one (RU38486 or Mifepestone); 11 $\beta$ -(4-dimethylaminophenyl)-17 $\alpha$ -hydroxy-17 $\beta$ -(3-hydroxypropyl)-13 $\alpha$ -methyl-4,9-gonadiene-3-one (ZK98299 or Onapristone); 11 $\beta$ -(4-acetylphenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(1-propinyl)-4,9-estradiene-3-one (ZK112993); 11 $\beta$ -(4-dimethylaminophenyl)-17 $\beta$ -hydroxy-17 $\alpha$ -(3-hydroxy-1 (Z)-propenyl-estra-4,9-diene-3-one (ZK98734); (7 $\beta$ 11 $\beta$ ,17 $\beta$ )-11-(4-dimethylaminophenyl)-7-methyl-4',5'-dihydrospiro ester-4,9-diene-17,2' (3'H)-furanl-3-one (Org31806); (11 $\beta$ ,14 $\beta$ ,17 $\alpha$ )-4',5'-dihydro-11-(4-dimethylamino-phenyl)y'spi roestra-4,9-diene-17,2' (3'H)-furanl-3-one (Org31376); 5-alpha-pregnane-3,2-dione. Additional non-natural ligands include, in general, synthetic non-steroidal estrogenic or anti-estrogenic compounds, broadly defined as selective estrogen receptor modulators (SERMS). Exemplary compounds include, but are not limited to, tamoxifen and raloxifen.

#### 4. Pharmaceutical compositions and combinations

Also provided is a pharmaceutical composition containing a therapeutically effective amount of the fusion protein, or a nucleic acid molecule encoding the fusion protein in a pharmaceutically acceptable carrier. Pharmaceutical compositions containing one or more fusion proteins with different zinc finger-nucleotide binding domains are contemplated. Also provided are pharmaceutical compositions containing the expression cassettes, and also compositions containing the ligands. Combinations containing a plurality of compositions are also provided.

#### Preparation of the compositions

The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well known. Typically such compositions are prepared as sterile injectables either as liquid

solutions or suspensions, aqueous or non-aqueous, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified. Tablets and other solid forms are contemplated.

5           The active ingredient can be mixed with excipients that are pharmaceutically acceptable and compatible with the active ingredient and in amounts suitable for use in the therapeutic methods described herein. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol or the like and combinations thereof. In addition, if  
10       desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, as well as pH buffering agents and the like which enhance the effectiveness of the active ingredient.

15           The therapeutic pharmaceutical composition can include pharmaceutically acceptable salts of the components therein. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide) that are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, tartaric, mandelic and the like. Salts formed with the free  
20       carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine and others.

          Physiologically tolerable carriers are well known in the art.  
25       Exemplary of liquid carriers are sterile aqueous solutions that contain no materials in addition to the active ingredients and water, or contain a buffer such as sodium phosphate at physiological pH value, physiological saline or both, such as phosphate-buffered saline. Still further, aqueous  
30       carriers can contain more than one buffer salt, as well as salts such as sodium and potassium chlorides, dextrose, propylene glycol, polyethylene glycol and other solutes.

Liquid compositions can also contain liquid phases in addition to and to the exclusion of water. Exemplary of such additional liquid phases are glycerin, vegetable oils such as cottonseed oil, organic esters such as ethyl oleate, and water-oil emulsions.

#### **D. Methods of gene regulation**

Method of regulating expression of endogenous and exogenous genes are provided. In particular, ligand-dependent methods are provided. In practicing the methods, a target nucleotide acid molecule containing a sequence that interacts with the nucleic acid binding domain of the fusion protein exposed to an effective amount of the fusion protein in the presence of an effective binding amount of a ligand, which can be added simultaneous with or subsequent to the fusion protein. The nucleic acid binding domain of the fusion protein binds to a portion of the target nucleic acid molecule and the ligand binds to the ligand binding domain of the fusion protein. Exposure can occur *in vitro*, *in situ* or *in vivo*.

The amount of zinc finger derived-nucleotide binding polypeptide required is that amount necessary to either displace a native zinc finger-nucleotide binding protein in an existing protein/promoter complex, or that amount necessary to compete with the native zinc finger-nucleotide binding protein to form a complex with the promoter itself. Similarly, the amount required to block a structural gene or RNA is that amount which binds to and blocks RNA polymerase from reading through on the gene or that amount which inhibits translation, respectively. Preferably, the method is performed intracellularly. By functionally inactivating a promoter or structural gene, transcription or translation is suppressed. Delivery of an effective amount of the inhibitory protein for binding to or "contacting" the cellular nucleotide sequence containing the zinc finger-nucleotide binding protein motif, can be accomplished by one of the mechanisms described herein, such as by retroviral vectors or liposomes, or other methods well known in the art.

In one embodiment, a method for inhibiting or suppressing the function of a cellular gene or regulatory sequence that includes a zinc finger-nucleotide binding motif. This is effected by contacting the zinc finger-nucleotide binding motif with an effective amount of a fusion protein that includes zinc finger-nucleotide binding polypeptide derivative that binds to the motif. In instances in which the cellular nucleotide sequence is a promoter, the method includes inhibiting the transcriptional transactivation of a promoter containing a zinc finger-DNA binding motif. The zinc finger-nucleotide binding polypeptide derivative may bind to a motif within a structural gene or within an RNA sequence.

### **Treatments**

Methods for gene therapy are provided. The fusion proteins are administered either as a protein or as a nucleic acid encoding the protein and delivered to cells or tissues in a mammal, such as a human. The fusion protein is targeted either to a specific sequence in the genome (an endogenous gene) or to an exogenously added gene, which is administered as part of an expression cassette. Prior to, simultaneous with or subsequent to administration of the fusion protein, a ligand that specifically interacts with the LBD in the fusion protein is administered. In embodiments, in which the targeted gene is exogenous, the expression cassette, which can be present in a vector, is administered, simultaneous with or subsequent to administration of the fusion protein. These methods are intended for treatment of any genetic disease, for treatment of acquired disease and any other conditions. Diseases include, cell proliferative disorders, such as cancer. Such therapy achieves its therapeutic effect by introduction of the fusion protein that includes the zinc finger-nucleotide binding polypeptide, either as the fusion or protein or encoded by a nucleic acid molecule that is expressed in the cells, into cells of animals having the disorder. Delivery of the fusion protein or nucleic acid molecule can be effected by any method known to those of skill in the art, including methods described herein. For example, it can

be effected using a recombinant expression vector such as a chimeric virus or a colloidal dispersion system.

The fusion proteins provided herein can be used for treating a variety of disorders. For example the proteins can be used for treating malignancies of the various organ systems, including but are not limited to, lung, breast, lymphoid, gastrointestinal, and genito-urinary tract adenocarcinomas, and other malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer, non-small cell carcinoma of the lung, cancer of the small intestine, and cancer of the esophagus. A polynucleotide encoding the zinc finger-nucleotide binding polypeptide is also useful in treating non-malignant cell-proliferative diseases such as psoriasis, pemphigus vulgaris, Behcet's syndrome, and lipid histiocytosis. Essentially, any disorder that is etiologically linked to the activation of a zinc finger-nucleotide binding motif containing promoter, structural gene, or RNA, would be considered susceptible to treatment with a polynucleotide encoding a derivative or variant zinc finger derived-nucleotide binding polypeptide.

The following examples are included for illustrative purposes only and are not intended to limit the scope of the invention.

#### EXAMPLE 1

##### **Construction and Testing of Designed Specific Zinc Finger Domains**

Variant zinc finger proteins have been designed and constructed to selectively bind to specific DNA sequences (Table 1). Table 1, below, summarizes the sequences (SEQ ID NO: 77-92) showing the highest selectivity for the sixteen embodiment of GNN target triplets.

**Table 1**

Target Specificity	Amino acids positions -1 1 2 3 4 5 6	SEQ ID NO:
GAA	<b>Q S S N L V R</b>	77
GAC	<b>D P G N L V R</b>	78
GAG	<b>R S D N L V R</b>	79
GAT	<b>T S G N L V R</b>	80
GCA	<b>Q S G D L R R</b>	81
GCC	<b>D C R D L A R</b>	82
GCG	<b>R S D D L V K</b>	83
GCT	<b>T S G E L V R</b>	84
GGA	<b>Q R A H L E R</b>	85
GGC	<b>D P G H L V R</b>	86
GGG	<b>R S D K L V R</b>	87
GGT	<b>T S G H L V R</b>	88
GTA	<b>Q S S S L V R</b>	89
GTC	<b>D P G A L V R</b>	90
GTG	<b>R S D E L V R</b>	91
GTT	<b>T S G S L V R</b>	92

#### **Oligonucleotides for zinc finger library panning**

Biotinylated, hairpin-structured target site oligos for panning of finger 2 ("F2") libraries had the following sequence:

##### **F2XXX:**

5'-Biotin-GGA CGC N'N'N' CGC GGG TTTT CCC **GCG NNN GCG** TCC-3' (SEQ ID NO: 25) where NNN = either of the 16 triplets of the GNN set, or TGA and N'N'N' = its complement.

Non-biotinylated, hairpin structured specific competitor oligos had the following sequence:

##### **F2NNN:**

5'-GGA CGC N'N'N' CGC GGG TTTT CCC **GCG NNN GCG** TCC-3' (SEQ ID NO: 25) where NNN = a mixture of all 64 existing triplets and N'N'N' = its complement.

### *Panning of zinc finger libraries*

Panning of zinc finger phage display libraries was carried out in solution using biotinylated target site hairpin oligos. Seven rounds of panning were carried out as follows: Phage prepared from an overnight culture was allowed to pre-bind to varying amounts of non-biotinylated specific competitor hairpin oligo prior to the addition of the target site oligo. The pre-binding was carried out in 400  $\mu$ l Zinc buffer A containing 1% Blotto, 5mM DTT, 4  $\mu$ g sheared herring sperm DNA and 100  $\mu$ l phage preparation. Typically, 10 times less specific competitor than target oligo was used for the first round of panning. For the subsequent panning rounds, the amount of specific competitor was gradually increased, up to a maximum of 12  $\mu$ g in the last panning round(s). Following 30 minutes at room temperature, 100  $\mu$ l Zinc buffer A containing 0.4  $\mu$ g biotinylated target hairpin oligo were added. After 2.5 to 3.5 hours at RT, phage bound to the target oligo was collected by the addition of 50  $\mu$ l Dynabeads M-280 suspension (Dyna) and incubation for one hour at RT. The beads were collected with a magnet, washed 10 times with Zinc buffer A (10 mM Tris, pH 7.5 / 90 mM KCl / 1 mM  $MgCl_2$  / 90  $\mu$ M  $ZnCl_2$ ) containing 2% Tween-20 and 5mM DTT, and once with Zinc buffer A containing 5mM DTT. Phage was eluted for 30 minutes at RT with 25  $\mu$ l of TBS containing 10 mg/ml trypsin. Following the addition of 75  $\mu$ l Super Broth, eluted phage was allowed to infect 5ml of *E. coli* ER2537 culture for 30 minutes in a 37 degrees Celsius shaker. The volume was increased to 10ml and Carbenicillin was added to a concentration of 20  $\mu$ g/ml. At this stage, the number of output phage was determined by plating aliquots of the infected bacteria onto Carbenicillin-containing LB-agar plates. After one hour shaking at 37 degrees Celsius, the Carbenicillin concentration was increased to 50  $\mu$ g/ml. After one more hour shaking at 37 degrees Celsius,  $10^{13}$  pfu helper phage was added and the culture was incubated for a few minutes at RT. Then, 90ml of Super Broth containing Carbenicillin (50  $\mu$ g/ml) and  $ZnCl_2$  (90  $\mu$ M) were added and the culture

was incubated at 37 degrees Celsius for two hours. Upon addition of Kanamycin to a final concentration of 70  $\mu$ g/ml, the culture was incubated in a 37 degrees Celsius shaker overnight. Phage was purified from culture supernatants by PEG precipitation and resuspended in 2 ml Zinc buffer A containing 1% BSA and 5 mM DTT for further rounds of panning. The number of phage was determined by using various dilutions of the phage prep for infection of *E. coli* ER2537, followed by plating onto Carbenicillin-containing LB-agar plates. Following seven rounds of panning, zinc finger cDNAs were subcloned into the bacterial expression vector pMal-CSS, a derivative of pMal-C2 (New England Biolabs), allowing for expression of the zinc finger proteins as maltose binding protein (MBP) fusions.

*Generation of proteins with desired DNA binding specificity.*

To generate DNA encoding three-finger proteins, F2 coding regions were PCR amplified from selected or designed F2 variants and assembled by PCR overlap extension. Alternatively, DNAs encoding three-finger proteins with a Zif268 or Sp1C framework were synthesized from 8 or 6 overlapping oligonucleotides, respectively. Sp1C framework constructs were generated as follows.

In the case of E2C-HS1(Sp1), 0.4 pmole each of oligonucleotides SPE2-3 (5'-GCG AGC AAG GTC GCG GCA GTC ACT AAA AGA TTT GCC GCA CTC TGG GCA TTT ATA CGG TTT TTC ACC-3' (SEQ ID NO: 26) and SPE2-4 (5'-GTG ACT GCC GCG ACC TTG CTC GCC ATC AAC GCA CTC ATA CTG GCG AGA AGC CAT ACA AAT GTC CAG AAT GTG GC-3') (SEQ ID NO: 27) were mixed with 40 pmole each of oligonucleotides SPE2-2 (5'-GGT AAG TCC TTC TCT CAG AGC TCT CAC CTG GTG CGC CAC CAG CGT ACC CAC ACG GGT GAA AAA CCG TAT AAA TGC CCA GAG-3') (SEQ ID NO: 28) and SPE2-5 (5'-ACG CAC CAG CTT GTC AGA GCG GCT GAA AGA CTT GCC ACA TTC TGG ACA TTT GTA TGG C-3') (SEQ ID NO:29) in a standard PCR mixture and cycled 25 times (30 seconds at 94 degrees Celsius, 30 seconds at 60 degrees

Celsius, 30 seconds at 72 degrees Celsius). An aliquot of this pre-assembly reaction was then amplified with 40 pmole each of the primers SPE2-1 (5'-GAG GAG GAG GAG GTG GCC CAG GCG GCC CTC GAG CCC GGG GAG AAG CCC TAT GCT TGT CCG GAA TGT GGT AAG TCC TTC TCT CAG AGC-3') (SEQ ID NO: 30) and SPE2-6 (5'-GAG GAG GAG GAG CTG GCC GGC CTG GCC ACT AGT TTT TTT ACC GGT GTG AGT ACG TTG GTG ACG CAC CAG CTT GTC AGA GCG-3') (SEQ ID NO: 31) using the same cycling conditions.

The E2C-HS2(Sp1), B3B-HS1(Sp1), B3B-HS2(Sp1), B3C2-HS1(Sp1), and B3C2-HS2(Sp1) DNAs were generated in the same way, using analogous sets of oligonucleotides differing only in the recognition helix coding regions. All assembled three-finger coding regions were digested with the restriction endonuclease *Sfi*I and cloned into pMal-CSS, a derivative of the bacterial expression vector pMal-C2 (New England Biolabs), allowing for expression of the zinc finger proteins as MBP fusions. DNAs encoding six-finger proteins with each of the different frameworks were assembled in pMal-CSS using *Xma*I and *Bsr*FI restriction sites included in the sequences flanking the three-finger coding regions (Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633).

#### Preparation of MBP-zinc finger fusion proteins for ELISA assays

Plasmid pMal constructs containing the zinc-finger coding sequences were transformed into the *E. coli* strain XL1-Blue by electroporation. Three milliliters of Super Broth were inoculated and grown overnight at 37 degrees Celsius. The next day, the cultures were diluted 1:20 in 50 ml conical tubes and grown at 37 degrees Celsius until  $OD_{600} = 0.5$ . IPTG was added to a final concentration of 0.3 mM, and incubation was continued for 2 hours. The cultures were centrifuged for 20 minutes, then the pellets resuspended in 400  $\mu$ l of Zinc Buffer A containing 5 mM fresh DTT. The samples were then frozen in dry ice/ethanol and thawed in 37 degrees Celsius water 6 times, then finally

centrifuged for 30 seconds and left on ice for 30 minutes before use of the supernatants.

#### *ELISA assays*

Streptavidin at a concentration of 0.2  $\mu\text{g}/25\mu\text{l}$  in PBS was added to each well of a 96 well plate, then incubated for 1 hour at 37 degrees Celsius. The plate was washed 2x with water, then biotinylated oligo at 0.1  $\mu\text{g}/25\mu\text{l}$  in PBS, or just PBS, was added to the appropriate wells and incubated for 1 hour at 37 degrees Celsius. The plate was washed 2x with water, then each well was filled with 3% BSA in PBS and incubated for 1 hour at 37 degrees Celsius. The BSA was removed without washing, and 25  $\mu\text{l}$  of the appropriate extract diluted in Zinc buffer A containing 5mM DTT was added to the appropriate wells. The binding reaction was allowed to proceed for 1 hour at room temperature. The plate was washed 8x with water, then  $\alpha$ -MBP mAb in Zinc buffer A and 1% BSA was added to the wells followed by incubation for 30 minutes at room temperature. The plate was washed 8x with water, then anti-mouse mAb conjugated to alkaline phosphatase in Zinc buffer A was added, and the plate was incubated for 30 minutes at room temperature. After 8 final washes with water, 25  $\mu\text{l}$  of alkaline phosphatase substrate and developer was added to each well. Incubation was performed at room temperature, and the OD<sub>405</sub> of each well was determined at 30 minute and 1 hour time points.

#### *Construction of zinc finger-transcription regulating domain fusion proteins*

cDNA encoding amino acids 473 to 530 of the *ets* repressor factor (ERF) repressor domain (ERD) (Sgouras *et al.* (1995) *EMBO J.* 14:4781-4793) was generated from four overlapping oligonucleotides using Taq DNA polymerase; a cDNA encoding amino acids 1 to 97 of the KRAB domain of KOX1 (Margolin *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:4509-4513) was assembled from 6 overlapping oligonucleotides; a cDNA encoding amino acids 1 to 36 of the Mad *sin3* interaction domain (SID) (Ayer *et al.* (1996) *Mol. Cell. Biol.* 16:5772-5781) was assembled

from 3 overlapping oligonucleotides. The coding region for amino acids 413 to 489 of the VP16 transcriptional activation domain (Sadowski *et al.*, (1988) *Nature* 335:563-564) was PCR amplified from pcDNA3/C7-C7-VP16 (Liu *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530).  
 5 The VP64 DNA, encoding a tetrameric repeat of VP16's minimal activation domain, comprising amino acids 437 to 447 (Seipel *et al.* (1992) *EMBO* 13:4961), was generated from two pairs of complementary oligonucleotides. All resulting effector domain-encoding fragments were fused to zinc finger coding regions by standard cloning procedures, such  
 10 that each resulting construct contained an internal SV40 nuclear localization signal, as well as a C-terminal HA decapeptide tag. Fusion constructs were cloned into pcDNA3 for expression in mammalian cells.

*Construction of integrin  $\beta$ 3 and erbB-2 luciferase reporter plasmids*

An integrin  $\beta$ 3 promoter fragment encompassing nucleotides -584  
 15 to -1 (with respect to the ATG codon) was PCR amplified from human genomic DNA, using the primers b3p(Nhe1)-f (5'-GAG GAG GAG GCT AGC GGG ATG TGG TCT TGC CCT CAA CAG GTA GG-3') (SEQ ID NO: 32) and b3p(Hind3)-b (5'-GAG GAG GAG AAG CTT CTC GTC CGC CTC CCG CGG CGC TCC GC-3') (SEQ ID NO: 33), and Taq Expand DNA  
 20 Polymerase mix (Boehringer). The cycling conditions were: 30 minutes at 94 degrees Celsius; 40 x (one minute at 94 degrees Celsius - 30 minutes at 62 degrees Celsius - 2.5 minutes at 72 degrees Celsius); 10 minutes at 72 degrees Celsius. 10% DMSO was present in the reaction mix.

An erbB-2 promoter fragment (Ishii *et al.* (1987) *Proc. Natl. Acad. Sci. U.S.A.* 84:4374-4378) encompassing nucleotides -751 to -1 was  
 25 PCR amplified under the same conditions, using the primers e2p(Nhe1)-f (5'-GAG GAG GAG GCT AGC CGA TGT GAC TGT CTC CTC CCA AAT TTG TAG ACC-3') (SEQ ID NO: 34) and e2p(Hind3)-b (5'-GAG GAG GAG AAG CTT GGT GCT CAC TGC GGC TCC GGC CCC ATG-3') (SEQ ID NO: 35). PCR products were purified with the Qiagen PCR prep kit, digested  
 30

with the restriction endonucleases Nhe1 and Hind3, and cloned into pGL3basic (Promega).

An *erbB*-2 promoter fragment encompassing nucleotides -1571 to -24 was excised from pSVOALΔ5'/*erbB*-2(N-N) by Hind3 digestion and subcloned into pGL3basic. pSVOALΔ5'/*erbB*-2(N-N) was a gift from Gordon Gill.

#### *Luciferase assays*

For all transfections, HeLa cells were plated in 24 well dishes and used at a confluency of 40-60%. Typically, 200 ng reporter plasmid (pGL3-promoter constructs or, as negative control, pGL3basic) and 20 ng effector plasmid (zinc finger constructs in pcDNA3 or, as negative control, empty pcDNA3) were transfected using the lipofectamine reagent (Gibco BRL). Cell extracts were prepared approximately 48 hours after transfection. Luciferase activity was measured with the Promega luciferase assay reagent, in a MicroLumat LB96P luminometer (EG&G Berthold).

#### *Selection strategy for the generation of six-finger proteins with DNA binding specificity*

Based on the modular nature of zinc finger domains, as well as the fact that each zinc finger recognizes 3 bp of DNA sequence, several strategies can be employed to generate zinc finger proteins, with preferably one to three fingers, with desired DNA binding specificity. For instance, *in vitro* evolution of a six-finger protein binding an 18bp target sequence can follow the strategy outlined in FIGURE 1. The target sequence is divided into six 3bp sub-sites, A-F. In the first step, a Zif268-based zinc finger phage display library in which the central finger 2 is randomized is selected against all 6 subsites in the context of the 2 wild type fingers. After successful generation of all the finger 2 variants required for a given target, cDNAs encoding three-finger proteins recognizing either half-site 1 (ABC) or half-site 2 (DEF) are constructed via PCR overlap extension. Finally, standard cloning procedures are used to

construct a gene encoding a six-finger protein recognizing the whole 18bp target site.

As an alternative to the serial connection of F2 domain variants, three-and six-finger proteins can be produced by "helix grafting". The framework residues of the zinc finger domains, those residues that support the presentation of the recognition helix, vary between proteins. The framework residues play a role in affinity and specificity. Thus, amino acid positions -2 to 6 of the DNA recognition helices are either grafted into a Zif268 (Pavletich *et al.* (1991) *Science* 252:809-817) or an Sp1C framework (Desjarlais *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:2256-2260).

#### *Choice of human integrin $\beta_3$ and erbB-2 target sequences*

Panning experiments carried out previously indicated that zinc fingers binding to G-containing triplets, with a G or a T in 5'-position, are more readily obtained than zinc fingers binding other triplets. The zinc finger target sequences were selected such that they contained one or more G's in each triplet of the 18bp sequence, and that each triplet started with a G or a T (Table 2). To conform with these requirements, *erbB-2* target B2 was split into two halves separated by two bases. A longer linker peptide between the appropriate zinc fingers may also for recognition of such a split site. Blast sequence similarity searches were carried out with each of the target sequences and confirmed that each 18bp sequence specifies a unique site in the human genome (maximal similarity tolerated: 16/18bp identity).

Since transcription factor AP-2 is involved in deregulated expression of *erbB-2* in a significant fraction of ErbB-2 overexpressing tumor cell lines, *erbB-2* target site B2 was designed to overlap with the AP-2 binding site GCTGCAGGC, with the intention of inhibiting expression of ErbB-2 not only as a result of active transcriptional repression, but also by competition with an important transcription factor. In contrast, zinc finger proteins binding the other *erbB-2* target sites (i.e.

*erbB-2* target sites C and D), affect transcription as a result of their effector domains.

Integrin  $\beta 3$  target sequences B and C2 were chosen at various distances from the transcription start site, to allow for a comparison of the efficacy of transcriptional regulation. Since the selected zinc finger proteins are fused to transcriptional effector domains (Sadowski *et al.*, (1988) *Nature* 335:563-564; Margolin *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:4509-4513) ; Sgouras *et al.* (1995) *EMBO* 14:4781-4793; Ayer *et al.* (1996) *Mol. Cell. Biol.* 16:5772-5781), binding of a zinc finger protein *per se* have an effect on the level of transcription.

A list of chosen target sequences for the selection of zinc finger proteins is given in Table 2, below. Since zinc finger proteins make base contacts predominantly with one strand of the DNA double helix, only the relevant strand of the target sequence is listed and designated +/- with respect to the coding strand. The location of the target sequences and position relative to the major transcription start site(s) is given.

Table 2: Chosen Target Sequences For The Selection Of Zinc Finger Proteins			
Integrin $\beta 3$ (B3) target sequences		LOCATION	SEQ ID
B3B	GCC TGA GAG GGA GCG GTG	- strand, promoter region, -160bp	72
B3C2	GGA GGG GAC GCG GTG GGT	- strand, promoter region, -70bp	73
<b>ErbB-2 (E2) target sequences</b>			
E2B2	GTG TGA GAA(CG)GCT GCA GGC	+ strand, promoter, -150/-220bp	74
E2B2	GTG TGA GAA(CG)GCT GCA GGC	+ strand, promoter, -150/-220bp	74
E2C	GGG GCC GGA GCC GCA GTG	+ strand, 5' UTR, +160/+230bp	75
E2D	GCA GTT GGA GGG GGC GAG	+ strand, promoter, -30/-100bp	76

#### *Construction and panning of a finger 2 library*

The amino acid residues implicated in contacting DNA in finger 2 of the Zif268-C7 ("C7") (Wu *et al.* (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92:344-348) have been extensively randomized using the PCR overlap extension mutagenesis strategy. Using two different randomization strategies, two sublibraries have been constructed using the pComb3

phage display vector (Barbas *et al.* (1991) *Proc. Natl. Acad. Sci. U.S.A.* 88:7978-7982). The sublibraries contain approximately  $4 \times 10^9$  independent clones each.

The mutagenesis strategy for randomization of finger 2 of Zif268-C7, showing helix positions -3 to 7, is summarized in Table 3, below. The top line shows the wild type sequence of finger 2. The lower two lines show the two mutagenesis strategies used, where N = G, A, T, C; K = G, T; V = G, A, C; S = G, C. The NNK randomized codon provides all 20 amino acids in 32 codons. The VNS randomized codon provides 16 amino acids in 24 codons, excluding Phe, Trp, Tyr, Cys and all stops. Note that in the strategy shown in the bottom line, the use of less complex codons allows for the mutagenesis of an additional codon.

**Table 3:**  
**Mutagenesis strategy for randomization of finger 2**  
**of Zif268-C7, showing helix positions -3 to 7.**

-3	-2	-1	1	2	3	4	5	6	7
F	S	R	S	D	H	L	T	T	H
F	S	(NNK)	(NNK)	(NNK)	(NNK)	L	(NNK)	(NNK)	H
F	(VNS)	(VNS)	(VNS)	(VNS)	(VNS)	L	(VNS)	(VNS)	H

Finger 2 variants recognizing each of the 16 triplets of the GXX set (Segal *et al.* (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96:2758-2763; and Table 1), as well as one variant recognizing TGA, have been successfully selected. In extension of previous observations, comparison of the zinc finger sequences revealed a code for zinc finger recognition of DNA. Thus, a 5'- and 3'-G selected an arginine at helix positions 6 and -1, respectively, while a central G selected an a histidine or lysine at position 3 of the recognition helix. In contrast, a central A selected an asparagine, a 3'-A a glutamine, a central T a serine or alanine, a 3'-T a threonine or serine, a central C an aspartate or threonine, and a 3'-C an aspartate or glutamate at the corresponding helix positions. An extensive characterization of the specificities and affinities of selected zinc finger variants has been carried out and indicates that many of the zinc finger

peptides recognize their targets in a highly specific manner (Segal *et al.* (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96:2758-2763 and Table 1).

*Refinement of finger 2 specificities by site-directed mutagenesis*

Attempts were made to improve binding specificity of some of the zinc finger domains by modifying the recognition helices by using site-directed mutagenesis. Data from the phage display selections and structural information guided the design of the mutants. Although helix positions 1 and 5 were not expected to play a direct role in recognition, the best improvements in specificity always involved modifications in these positions (Segal *et al.* (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96:2758-2763 and Table 1). These residues have been observed to make phosphate backbone contacts, which contribute to affinity in a nonsequence-specific manner. Thus, removal of nonspecific contacts can increase the importance of the specific contacts to the overall stability of the complex, thereby enhancing specificity.

*Generation of three finger proteins binding *erbB-2* and integrin  $\beta 3$  target sequences*

Two different strategies for generating three-finger proteins recognizing 9 bp of DNA sequence were used. Each strategy is based on the modular nature of the zinc finger domain, and takes advantage of a family of zinc finger domains recognizing triplets of the 5'-(GNN)-3' type defined in Table 1. Two three-finger proteins recognizing half sites (HS) 1 and 2 of the 5'-(GNN)<sub>3</sub>-3' *erbB-2* target site e2c were generated in the first strategy by fusing the pre-defined finger 2 (F2) domain variants together using a PCR assembly strategy.

To examine the generality of this approach, three additional three-finger proteins recognizing sequences of the 5'-(GNN)<sub>3</sub>-3' type, were prepared using the same approach. Purified zinc finger proteins were prepared as fusions with the maltose binding protein (MBP). ELISA analysis revealed that serially connected F2 proteins were able to act in concert to specifically recognize the desired 9-bp DNA target sequences

(Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). Each of the 5 proteins shown was able to discriminate between target and non-target 5'-(GNN)<sub>3</sub>-3' sequence. The affinity of each of the proteins for its target was determined by electrophoretic mobility-shift assays. These studies demonstrated that the zinc finger peptides have affinities comparable to Zif268 and other natural transcription factors with  $K_d$  values that ranged from 3 to 70 nM (Table 4, below).

As an alternative to the serial connection of F2 domain variants, in the second strategy, three-finger proteins specific for the two halvesites of the *erbB-2* target site e2c (Table 4, below), were produced by "helix grafting." The framework residues may play a role in affinity and specificity. For helix grafting, amino acid positions -2 to 6 of the DNA recognition helices were either grafted into a Zif268 (Pavletich *et al.* (1991) *Science* 252:809-817) or an Sp1C framework (Desjarlais *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:2256-2260). The Sp1C protein is a designed consensus protein shown to have enhanced stability towards chelating agents. The proteins were expressed from DNA templates prepared by a rapid PCR-based gene assembly strategy. In each case, ELISA analysis of MBP fusion proteins showed that the DNA binding specificities and affinities (Table 4, below) observed with the F2 framework constructs were retained. Three finger proteins recognizing HS1 and HS2 of the integrin  $\beta 3$  target sites b3b and b3c2 have also been generated, using the Sp1C backbone. Preliminary ELISA data showed that these proteins bind their respective targets with good specificity. Further characterization of proteins can be made, such as determination of their affinities by gel shift analysis. See Table 4, below.

*Generation of six-finger proteins for specific targeting of the erbB-2 and integrin  $\beta 3$  promoter regions.*

The recognition of 9 bp of DNA sequence is not sufficient to specify a unique site within a complex genome. In contrast, a six-finger protein recognizing 18 bp of contiguous DNA sequence could define a

single site in the human genome, thus fulfilling an important prerequisite for the generation of a gene-specific transcriptional switch. Six-finger proteins binding the *erbB-2* target sequence e2c were generated from three-finger constructs by simple restriction enzyme digestion and cloning with F2, Zif268, and Sp1C framework template DNAs (for sequences of these proteins, see Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). Six finger proteins binding the integrin  $\beta 3$  target sequences b3b and b3c2 were only generated using the Sp1C backbone. ELISA analysis of purified MBP fusion proteins showed that each of the six-finger proteins was able to recognize the specific target sequence, with little cross reactivity to non-target 5'-(GNN)<sub>6</sub>-3' sites or a tandem repeat of the Zif268 target site.

In Table 4, below, the affinities of three- and six-finger proteins for various target sequences as determined by gel shift analysis is summarized. Proteins are named with upper case letters, DNA target sequences with lower case letters. Abbreviations used are: F2 = finger 2 framework; Zif = Zif268 framework; Sp1 = Sp1C framework; mut = mutant; HS = half-site. With respect to the target site overlap phenomenon, the base following each target sequence is given in lower case letter (see Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). The affinity of the Zif268-DNA interaction was determined to be 10 nM (Segal *et al.* (1999) *Proc. Natl. Acad. Sci. U.S.A.* 96:2758-2763).  $K_d$  values are averages from 2 independent experiments, with standard deviations of 50% or less.

Table 4:  
AFFINITIES OF THREE AND SIX FINGER PROTEINS

Protein	Target	Target Sequence (5'-3')	K <sub>d</sub> , nM
B3(F2)	b3	GGA GGG GAC g	4
E2(F2)	e2	GGG GGC GAG g	3
C5(F2)	c5	GGA GGC GGG g	30
E2C- HS1(F2)	e2c-hs1	GGG GCC GGA g	45
E2C- HS1(Zif)	e2c-hs1	GGG GCC GGA g	70
E2C- HS1(Sp1)	e2c-hs1	GGG GCC GGA g	35
E2C- HS2(F2)	e2c-hs2	GCC GCA GTG g	70
E2C- HS2(Zif)	e2c-hs2	GCC GCA GTG g	75
E2C- HS2(Sp1)	e2c-hs2	GCC GCA GTG g	25
E2C(F2)	e2c-g	GGG GCC GGA GCC GCA GTG g	25
E2C(Zif)	e2c-g	GGG GCC GGA GCC GCA GTG g	1.6
E2C(Zif)	e2c-a	GGG GCC GGA GCC GCA GTG a	2.3
E2C(Zif)	e2c-muths1	AGT CTG AAT GCC GCA GTG g	200
E2C(Zif)	e2c-muths2	GGG GCC GGA AGT CTG AAT g	200
E2C(Sp1)	e2c-g	GGG GCC GGA GCC GCA GTG g	0.5
E2C(Sp1)	e2c-a	GGG GCC GGA GCC GCA GTG a	0.75
E2C(Sp1)	e2c-muths1	AGT CTG AAT GCC GCA GTG g	65
E2C(Sp1)	e2c-muths2	GGG GCC GGA AGT CTG AAT g	100

In Table 5, below, the finger 2 variants generated by phage display selection and refined by site-directed mutagenesis are summarized.

Protein designations are in the form pXXX, for clones derived from panning; pmXXX refers to clones refined by mutagenesis. Helix positions -1, 3, and 6 are shown in **bold**, altered nucleotides are underlined. The values represent the results of at least two independent experiments. The standard error was  $\pm 50\%$  or less.

**Table 5:**  
**SUMMARY OF FINGER 2 VARIANTS GENERATED BY PHAGE DISPLAY**  
**SELECTION AND REFINED BY SITE-DIRECTED MUTAGENESIS**

	Protein	Finger-2 Helix	Finger-2 Subsite	$K_D$ (nM)	$K_{D, \text{Prot}}/K_{D, \text{Zif268}}$
5	pGGG	SRSDHLTR	GGG	0.4	0.04
	pmGGG	SRSDKLVR	GGG	6	0.6
	"	"	G <u>T</u> G	> 1,400	
	pGGA	SQRAHLER	GGA	3	0.3
	pmGGT	STSGHLVR	GGT	15	1.5
10	"	"	G <u>G</u> C	> 2,400	
	pmGGC	SDPGHLVR	GGC	40	4.0
	pmGAG	SRSDNLVR	GAG	1	0.1
	"	"	G <u>G</u> G	45	4.5
	pmGAA	SQSSNLVR	GAA	0.5	0.05
15	pGAT	STSGNLVR	GAT	3	0.3
	pmGAC	SDPGNLVR	GAC	3	0.3
	"	"	G <u>C</u> C	90	9.0
	pGTG	SRKDSLVR	GTG	3	0.3
	pmGTG	SRSDELVR	GTG	15	1.5
20	"	"	G <u>A</u> G	30	3.0
	pGTA	SQSSSLVR	GTA	25	2.5
	"	"	G <u>T</u> G	> 1,000	
	pmGTT	STSGSLVR	GTT	5	0.5
	pGTC	SDPGALVR	GTC	40	4.0
25	"	"	G <u>C</u> C	> 4,400	
	pmGCG	SRSDDLVR	GCG	9	0.9
	"	"	G <u>A</u> G	6	0.6
	pGCA	SQSGDLRR	GCA	2	0.2
	"	"	G <u>C</u> T	10	1
30	pmGCT	STSGELVR	GCT	65	6.5
	pGCC	SDCRDLAR	GCC	80	8.0
	pTGA	SQAGHLAS	TGA	nd	nd
	C7	SRSDHLTT	TGG	0.5	0.05
35	Zif268	SRSDHLTT	TGG	10	1

The affinity of each of the E2C proteins for the e2c DNA target site was determined by gel-shift analysis. A modest  $K_d$  value of 25 nM was observed with the E2C(F2) six-finger protein constructed from the F2

framework (Table 5, above; Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633), a value that is only 2 to 3 times better than its constituent three-finger proteins. In previous studies of six-finger proteins, an approximately 70-fold enhanced affinity of the six-finger proteins for their DNA ligand compared to their three-finger constituents was observed (Liu *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:5525-5530). The absence of a substantial increase in the affinity of the E2C(F2) peptide suggested that serial connection of F2 domains is not optimal. It is possible that the periodicity of the F2 domains of the six-finger protein does not match that of the DNA over this extended sequence, and that a significant fraction of the binding energy of this protein is spent in unwinding DNA. In contrast to the F2 domain protein, the E2C(Zif) and E2C(Sp1) six-finger proteins displayed 40- to 70-fold increased affinity as compared to their original three-finger protein constituents, with  $K_d$  values of 1.6nM and 0.5nM, respectively. Significantly, both three-finger components of these proteins were involved in binding, since mutation of either half-site led to a roughly 100-fold decrease in affinity (Table 4, above; Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). The preponderance of known transcription factors bind their specific DNA ligands with nanomolar affinity, suggesting that the control of gene expression is governed by protein/DNA complexes of unexceptional life times. Thus, zinc finger proteins of increased affinity should not be required and could be disadvantageous, especially if binding to non-specific DNA is also increased. The affinities of the B3B(Sp1) and B3C2(Sp1) six finger proteins for their respective targets can be determined by one skilled in the art using well-known methods as well as those described herein.

## EXAMPLE 2

**Construction Of Fusion Proteins Containing Zinc Finger Domains and Transcriptional Repressors And Activators**

In order to demonstrate use of zinc finger proteins as gene-specific transcriptional regulators, the E2C(Sp1), B3B(Sp1), and B3C2(Sp1) six-finger proteins were fused to a number of effector domains (Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). Transcriptional repressors were generated by attaching either of three human-derived repressor domains to the zinc finger protein. The first repressor protein was prepared using the ERF repressor domain (ERD) (Sgouras *et al.* (1995) *EMBO J.* 14:4781-4793), defined by amino acids 473 to 530 of the ets2 repressor factor (ERF). This domain mediates the antagonistic effect of ERF on the activity of transcription factors of the *ets* family. A synthetic repressor was constructed by fusion of this domain to the C-terminus of the zinc finger protein.

The second repressor protein was prepared using the Krüppel-associated box (KRAB) domain (Margolin *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:4509-4513). This repressor domain is commonly found at the N-terminus of zinc finger proteins and presumably exerts its repressive activity on TATA-dependent transcription in a distance- and orientation-independent manner, by interacting with the RING finger protein KAP-1. The KRAB domain found between amino acids 1 and 97 of the zinc finger protein KOX1 was used. In this case an N-terminal fusion with the six-finger protein was constructed. Finally, to demonstrate the utility of histone deacetylation for repression, amino acids 1 to 36 of the Mad mSIN3 interaction domain (SID) were fused to the N-terminus of the zinc finger protein (Ayer *et al.*, (1996) *Mol. Cell. Biol.* 16:5772-5781). This small domain is found at the N-terminus of the transcription factor Mad and is responsible for mediating its transcriptional repression by interacting with mSIN3, which in turn interacts the co-repressor N-CoR and with the histone deacetylase mRPD1.

To examine gene-specific activation, transcriptional activators were generated by fusing the zinc finger protein to amino acids 413 to 489 of the herpes simplex virus VP16 protein (Sadowski *et al.* (1988) *Nature* 335:563-564), or to an artificial tetrameric repeat of VP16's minimal activation domain, DALDDFDLML (SEQ ID NO: 36) (Seipel *et al.* (1992) *EMBO* 13:4961), designated VP64.

*Specific regulation of erbB-2 promoter activity*

Reporter constructs containing fragments of the *erbB-2* promoter coupled to a luciferase reporter gene were generated to test the specific activities of the *erbB-2* specific synthetic transcriptional regulators. The target reporter plasmid contained nucleotides -758 to -1 with respect to the ATG initiation codon, whereas the control reporter plasmid contained nucleotides -1571 to -24, thus lacking all but one nucleotide of the E2C binding site encompassed in positions -24 to -7. Both promoter fragments displayed similar activities when transfected transiently into HeLa cells, in agreement with previous observations. To test the effect of zinc finger-repressor domain fusion constructs on *erbB-2* promoter activity, HeLa cells were transiently co-transfected with each of the zinc finger expression vectors and the luciferase reporter constructs (Beerli *et al.*, (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633). Significant repression was observed with each construct. The ERD and SID fusion proteins produced approximately 50% and 80% repression, respectively. The most potent repressor was the KRAB fusion protein. This protein caused complete repression of *erbB-2* promoter activity. The observed residual activity was at the background level of the promoter-less pGL3 reporter. In contrast, none of the proteins caused significant repression of the control *erbB-2* reporter construct lacking the E2C target site, demonstrating that repression is indeed mediated by specific binding of the E2C(Sp1) protein to its target site. Expression of a zinc finger protein lacking any effector domain resulted in weak repression, approximately 30%, indicating that most of the repression observed with the SID and

KRAB constructs is caused by their effector domains, rather than by DNA-binding alone. This observation strongly suggests that the mechanism of repression is active inhibition of transcription initiation rather than of elongation. Once initiation of transcription by RNA polymerase II has occurred, the zinc finger protein appears to be readily displaced from the DNA by the action of the polymerase.

The use of *erbB-2* specific zinc finger proteins to mediate activation of transcription was demonstrated using the same two reporter constructs. The VP16 fusion protein was found to stimulate transcription approximately 5-fold, whereas the VP64 fusion protein produced a 27-fold activation. This dramatic stimulation of promoter activity caused by a single VP16-based transcriptional activator is exceptional in view of the fact that the zinc finger protein binds in the transcribed region of the gene. This again demonstrates that mere binding of a zinc finger protein, even with one with sub-nanomolar affinity, in the path of RNA polymerase II need not necessarily negatively affect gene expression.

Based on the efficient and specific regulation of a reporter construct driven by the *erbB-2* promoter, the effect of transiently transfected zinc finger expression plasmids on activity of the endogenous *erbB-2* promoter was analyzed. As a read-out of *erbB-2* promoter activity, ErbB-2 protein levels were analyzed by Western blotting. Significantly, E2C(Sp1)-VP64 lead to an upregulation of ErbB-2 protein levels, while E2C(Sp1)-SKD lead to its downregulation. This regulation was specific, since no effect was observed on expression of EGFR.

It is important to note that the observations made in these experiments drastically underestimate the efficacy of the zinc finger peptides, since the transfection efficiency of HeLa cells is no more than 50%. To ascertain that 100% of the cells express the zinc finger proteins stable cell lines need to be generated. Production of stable cell lines expressing the zinc finger constructs under control of a tetracycline-

inducible promoter is known (Gossen *et al.* (1992) *Proc. Natl. Acad. Sci. U.S.A.* 89:5547-5551). Inducible expression of zinc finger proteins in stable cell lines allows for detailed analysis of the degree of specificity of such proteins.

#### Specific regulation of integrin $\beta 3$ promoter activity

To test the activity of transcriptional regulators specific for the integrin  $\beta 3$  promoter, a reporter plasmid was constructed containing the luciferase open reading frame under control of the integrin  $\beta 3$  promoter. When compared to the two *erbB-2* promoter fragments described above, the integrin  $\beta 3$  promoter fragment had a very low activity. In fact, in some experiments no activation of luciferase expression over background was detected, preventing an analysis of the effects of the KRAB fusion proteins. However, when the VP64 fusion proteins were tested an efficient activation of the integrin  $\beta 3$  promoter was observed. B3B(Sp1)-VP64 and B3C2(Sp1)-VP64 stimulated transcription 12 and 22fold, respectively. Activation of transcription was specific, since no effect on the activity of the *erbB-2* promoter was detected.

### EXAMPLE 3

#### Fusion Protein Construct Comprising Progesterone Receptor Variant

Amino acid sequence comparisons of steroid receptor family members indicates that they generally comprise a number of defined domains, including an N-terminal DNA binding domain and a more C-terminally located ligand binding domain. Importantly, these domains are modular and the DNA binding domain of progesterone receptor (PR) has been successfully exchanged for the Gal4 DNA binding domain. The addition of a VP16 activation or a KRAB repressor domain to the N- or C-terminus of this construct yielded proteins that could regulate a Gal4 responsive reporter in a ligand dependent manner. An important feature of the ligand binding domain used in these studies is that it is derived from a mutant PR with a small C-terminal deletion. This mutant fails to respond

to progesterone and is responsive only to progesterone antagonists such as RU486, making this system suitable for *in vivo* applications.

The original PR DNA binding domain can be replaced by engineered zinc finger proteins. For example, the three finger protein Zif268(C7) was fused to the N-terminus of the PR ligand binding domain (PBD) (aa 640 to 914), and the VP16 activation domain to its C-terminus. It was found that this fusion protein was able to regulate an SV40 promoter luciferase construct with ten upstream Zif268(C7) binding sites in an RU486-dependent manner.

An RU486 dose response curve showed that optimal induction occurs at about 1nM to about 10nM RU486. A time course study was carried out with 10nM RU486 and showed that optimal induction of C7-PBD-VP16 activity occurs at about 24 hours.

Since naturally occurring steroid receptors bind DNA as dimers, an important prerequisite for the application of this approach is the presence of suitable target sequences in the promoter of interest. Fortunately, the spacing and orientation of the two half-sites targeted by steroid receptor dimers is flexible. While a steroid response element usually includes an inverted repeat, or palindrome, also direct repeats or even everted repeats of the half-sites in variable spacing are tolerated (Aumais *et al.* (1996) *J. Biol. Chem.* 272:12229-12235). A search of the *erbB-2* and integrin  $\beta 3$  promoters revealed that direct and inverted repeats of 5'-(GNN)<sub>3</sub>-3' sequence motifs occur quite frequently. An example of a sequence motif suitable for targeting by a heterodimeric RU486-regulatable zinc finger protein is 5' **GAG GAG GGC** TGCTT **GAG GAA GTA**-3' (SEQ ID NO: 37), which was found in the *erbB-2* promoter and overlaps with the TATA box (underlined above). In some instances, promoter targeting is possible using a homodimer, for example by targeting the sequence 5'-**GCC GGA GCC ATGGG GCC GGA GCC**-3' (SEQ ID NO: 38), which is also found in the *erbB-2* promoter and overlaps with the target sequence e2c (underlined).

#### EXAMPLE 4

##### **Recombinant Ligand Activated Transcriptional Regulator Fusion Proteins Containing Human Estrogen Receptor Ligand Binding Domains**

The human estrogen receptor is shown in FIG. 2 as an example of a steroid receptor protein. The numbers below the rectangle indicate the position of the amino acid residues defining the borders of each domain. A/B is the domain of the amino terminus activation function 1 (AF-1), C is the DNA binding domain, D is called the hinge region, E is the ligand binding domain, which also contains the activation function 2 (AF-2) and F is the portion closest the carboxyl terminal, a domain whose function has not been fully established. The regions of the protein that participate and stabilize the homodimerized complex are distributed in the C, D and E domains. Regions throughout the steroid receptor ligand binding domain (region E in FIG. 2) as well as regions in the native DBD and hinge region (regions C and D respectively) contribute to homodimerization of the receptor. To demonstrate the importance of these regions to the function of the C2H2-containing receptors, proteins containing three different length LBD fragments were constructed. These differing length LBD constructs are designated A, B, and C (FIG. 3). LBD fragment A represents what is generally referred to as the "minimal" LBD fragment. Some studies have suggested the hinge region plays an important role in steroid receptor LBD — chimeric proteins; fragment B represents the LBD plus hinge. The native C or DNA binding region of estrogen receptor contains two zinc fingers of the C4-C4 class. The 5' or amino terminus finger contributes to DNA specific contacts; the 3' finger contributes to stabilizing the DNA binding domain dimer complex. To take advantage of this contribution of the 3' native zinc finger, LBD fragment C, where the 3' native zinc finger is retained and fused directly to the C2H2 zinc finger array, was included.

In order to optimize the ability of the fusion proteins to regulate gene expression, it may be necessary to add additional heterologous

transactivating domains to the receptor. To facilitate these studies, fusion proteins were constructed either with the full length LBD extending to estrogen receptor residue 595, or with LBD fragments truncated at amino acid (aa) 554 to remove the F region. The full-length constructs are referred to as long (L), the truncated versions as short (S). All constructs contain a heterologous transactivation domain (TA) comprised of a VP16 minimal domain, unless otherwise noted, fused to the carboxy terminus of the ligand binding domain. VP16 minimal domain trimer has the amino acid residue sequence 3 x (PADALDDFDLDM) (SEQ ID NO: 36), and is the tetracycline controlled transactivator (tTA) TA2 (Baron *et al.* (1997) *Nucleic Acids Research* 25:2723-2729).

These constructs are summarized in FIG. 3, which provides a schematic summary of the cloning strategy and nomenclature related to the C2H2 DNA binding domain - ER ligand binding domain fusion proteins. As shown in the plasmid construct at the bottom, the final construct contains three components: a C2H2 zinc finger domain (ZFP) at the amino end, a steroid receptor ligand binding domain (LBD) fragment in the middle, and a heterologous transactivation domain (TA) appended onto the carboxyl end. LBD fragments A, B, or C were defined by the position of the amino terminus border of the LBD; amino acid number for A (283), B (258) and C (212) correspond to the residue numbers in wild type ER. LBD fragments were further defined as long (L) or short (S) depending on their carboxy terminus junction. Long constructs fuse the heterologous TA to the wt ER amino acid residue 595, short constructs fuse TA to an LBD fragment truncated at ER amino acid 554. Thus, six fusion proteins in all were constructed, ZFP-LBD-TA A, B and C, each in a long and short form. Maps of specific examples constructed in the expression vector pcDNA3.1 are shown in FIG. 4 (C7LBDAS) (SEQ ID NO: 6), FIG. 5 (C7LBDDBS) (SEQ ID NO: 8), FIG. 6 (C7LBDACS) (SEQ ID NO: 10), FIG. 7 (C7LBDAL) (SEQ ID NO: 7), FIG. 8 (C7LBDDBL) (SEQ ID NO: 1), and FIG. 9 (C7LBDACL) (SEQ ID NO: 9).

As discussed in detail above, zinc fingers of the C2H2 class each contribute to about 3 bp of DNA sequence contacts. C2H2 zinc finger arrays can be "stitched together" to assemble DNA binding domains having 6, 9, 12, 15, 18 bp or more of specific sequence to which they bind. In order to evaluate the size of the zinc finger array that can be used in these C2H2 Zn finger (ZFP)— steroid receptor fusion proteins, proteins containing 3 finger and 6 finger arrays were constructed. The composition of the various proteins assembled, and their DNA binding site specificity is listed in FIG. 16.

The general cloning strategy was as follows. Three fragments (A, B, and C with reference to FIG. 3) of human estrogen receptor ligand binding domain (LBD) with or without the F region were built into the pcDNA3.1 (Invitrogen) vector backbone through a series of PCR amplification and cloning steps. Initially the LBD fragment A without F region (i.e. short form; LBDAS) and with F region (i.e. long form; LBDAL) were PCR amplified from a plasmid clone of the human wild type estrogen receptor, pHEGO (Tora *et al.* *EMBO J.* 8:1981-1986) with primer pairs NR1/NR2 and NR1/NR3 respectively (Table 1). Convenient restriction sites were incorporated into primers (Table 1) as needed. The PCR amplified LBDAS and LBDAL fragments were first cloned into the SrfI site of pCR-ScriptAmpSK(+) vector (Stratagene), resulting in constructs pLBDAS and pLBDAL. The VP16 minimal domain trimer (TA2; Baron *et al.* (1997) *Nucleic Acids Research* 25:2723-2729) was PCR amplified from plasmid pTTA2 (Clontech) with primer pairs NR4 and NR9 and cloned into the SplI and NotI site of pLBDAS and pLBDAL to generate pLBDASTA2 and pLBDALTA2. To generate LBD fragment B without the F region (LBDBS) and LBD fragment C without the F region (LBDACS), PCR primers NR7 and NR8, which represent the 5' boundary of the LBD region fragment in chimerics B and C respectively were designed (Table 6, below). These primers were paired with the 3' end primer NR6, which incorporates a unique BspI site in ER. PCR fragments from pHEGO with

primer pair NR6/NR7 and PCR fragment with NR6/NR8 were then cloned into the SpeI and BipI site of pLBDC7ASTA2 backbone. This resulted in plasmid pLBDBSTA2 and pLBDCSTA2.

**Table 6**  
**PCR Primers Used For Cloning**

NAME / (SEQ ID NO:)	SEQUENCE
NR1 (39)	cct act gcc ggc act agt tct gct gga gac atg aga gct gcc aac ctt
NR2 (40)	cct aaa cgt acg gct agt ggg cgc atg tag gcg gtg ggc gtc
NR3 (41)	cct aaa cgt acg gac tgt ggc agg gaa acc ctc tgc ctc
NR4 (42)	cca ctt aaa tgt gaa agt cgt acg ccg gcc
NR6 (43)	tat ggg ggg ctc agc atc caa caa ggc act
NR7 (44)	cct act act agt gac cga aga gga ggg aga atg ttg aaa cac aag cgc
NR8 (45)	cct act act agt agt att caa gga cat aac gac tat atg tgt
NR9 (46)	tat cat gtg cgg ccg ctt act tag tta ccc cgg cag cat

Having completed cloning of the three LBD fragments fused to the TA2 region, the C2H2 DNA binding protein C7 was then excised from pcDNAC7VP16 by BglII and SpeI digestion and ligated into the BamHI and SpeI site of each of the 3 constructions (pLBDA2A2, pLBDBSTA2 and pLBDCSTA2), which resulted in pC7LBDA2A2, pC7LBDBSTA2 and pC7LBDCSTA2. Cassettes of C7LBDA2A2, C7LBDBSTA2 and C7LBDCSTA2 were then removed from the pCR-Script vector by EcoRI-NotI digestion and cloned into the same sites of the expression cassette vector pcDNA3.1(+), resulting in constructs pCDNAC7ASTA2, pCDNAC7BSTA2 and CDNAC7CSTA2. In order to reconstruct these three ZFP-LBD fusion proteins with an LBD fragment including the estrogen receptor F region fused to TA2, the BipI to NotI fragment was excised from pLBDA2A2 construct and substituted for the BipI-NotI fragment in pCDNAC7LBDA2A2, pCDNAC7LBDBSTA2 and pCDNAC7LBDCSTA2 to generate pCDNAC7LBDA2A2, pCDNAC7LBDBLTA2 and pCDNAC7LBDCSTA2.

### *Cloning for Replacement of DNA Binding Domain C7 with E2C*

An intermediate construct pcDNAE2CVP16 was first constructed by replacing the *Sfi*I fragment containing C7 in pcDNAC7VP16 with the E2C(hs1) fragment isolated from pMal/E2C(hs1) after *Sfi*I digestion.

Next, pcDNAE2CVP16 was digested with *Spe*I and a 1 kb fragment was isolated. This *Spe*I fragment was ligated to the large *Spe*I fragment of pcDNAC7LBDASTA2, which created pcDNA-E2CLBDASTA2. Similar steps were performed to construct pcDNAE2CLBDBSTA2.

### *Analysis of Recombinant Construct Protein Binding to DNA*

In order to demonstrate that the fusion proteins bind to DNA in a sequence specific manner, and to evaluate the stoichiometry of protein:DNA binding, standard electrophoretic mobility shift or gel retardation assays were performed.

First, fusion proteins were produced by *in vitro* transcription and translation using the TNT Coupled Reticulocyte Lysate System (Promega, Cat # L4610) according to the manufacturer's instructions. Briefly, each expression reaction was set up in a total volume of 50  $\mu$ l which contained 25  $\mu$ l of TNT rabbit reticulocyte lysate, 2  $\mu$ l of TNT Reaction Buffer, 2  $\mu$ l of RNasin ribonuclease inhibitor (20 U/ $\mu$ l), 1  $\mu$ l each of amino acid mixture minus leucine, amino acid mixture minus methionine and TNT T7 RNA polymerase, 2  $\mu$ l of expression plasmid (1 $\mu$ g/ $\mu$ l) and water. The reaction mixture was incubated at 30° C for 90 minutes.

Binding of the expressed protein to duplex oligonucleotides was performed as follows, using the gel shift assay systems (Promega, Cat # E3050): 5  $\mu$ l of *in vitro* translation product was co-incubated with 4  $\mu$ l of 5X gel shift binding buffer and 7  $\mu$ l of water at room temperature for 20 min, then 2  $\mu$ l of E2 (10 nM final concentration) and 2  $\mu$ l of <sup>32</sup>P-labeled probe were added to the mixture. The probe had been labeled using standard protocol as described in the kit. After incubated at room temperature for about 20 minutes, the mixture was loaded onto a 6%

DNA retardation gel and run in 0.5X TBE buffer at 150-200 volts for about 30-60 minutes. The gel was then dried and exposed to X-ray film.

5 A DNA oligonucleotide containing two inverted binding sites for the C2H2 domain known as C7, each half site separated by 3 bp, was used for the initial assessment of DNA binding. This palindromic configuration mimics the composition of the native estrogen receptor response element (ERE), except that the natural 6 bp half site of ERE is replaced by the 9 bp half site specified by C7. Binding of the C7-LBD fusion proteins A, B, and C, all in the short form, were tested and compared to the control proteins C7VP16 and 2C7VP16 (see, Liu, *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A* 94:5525-5530, which describes the control proteins). For each protein, binding was tested in the absence or presence of 100 fold excess of unlabeled oligonucleotide (1.75  $\mu$ M) as a competitor. Competition of the gel shift product by the unlabeled oligonucleotide indicates the band is a specific protein:DNA interaction. The results demonstrated that C7VP16 can bind once or twice to the oligonucleotide, creating two specific gel shift bands. 2C7VP16 binds only once to the oligonucleotide containing two inverted C7 sites. Notably, C7LBDA and C7LBDB bind strongly to yield one major species, which runs higher than any of the control bands. Although true molecular mass cannot be determined from this type of mobility assay, the relative size of the complexes suggest the protein bound for C7LBD is larger than for C7VP or 2C7VP. The size of the band and presence of only one major species indicate that the fusion protein ZFP-LBD is binding to the oligonucleotide as a dimer. No significant gel shift product was detected for C7LBD chimeric C, suggesting that the addition of the additional native zinc finger from the estrogen receptor may have reduced the affinity of the fusion protein for its C2H2-specific DNA binding site. Finally, the reduction of binding for each of the gel shift products by the addition of the unlabeled oligonucleotide indicates that these fusion proteins are binding to DNA in a sequence specific manner.

To further demonstrate that the chimera ZFP-LBD binds to DNA as a dimer, the binding of C7LBD A, B, and C to oligonucleotides containing one or two C7 binding sites was tested. Three fusion proteins (C7LBDDAS, C7LBDBS and C7LBDCS) were tested against three different target oligonucleotide sequences, which contained one C7 half site or two C7 half sites either in palindromic or direct repeat orientation.

Oligo 1: gat cca aag tcg cgt ggg cgc agc gcc cac gcg atc aaa ga (SEQ ID NO: 48)

Oligo2: gat cca aag tcc agg cga gcg cgt ggg cgg cag atc aaa ga (SEQ ID NO: 49)

Oligo3: gat cca aag tcg cgt ggg cgc agg cgc gag cgt ggg cgg atc aaa ga (SEQ ID NO: 50) Gel shift assay conditions were the same as the standard protocol described above. The results showed that C7LBDDAS and C7LBDBS were able to bind to both oligonucleotides containing two C7 half sites, but not to the oligo containing only one half site. C7LBDCS bound weakly or not at all to all three targets.

Fusion proteins C7LBDA and C7LBDB bound to the probe containing a palindrome (two inverted half sites) as a single form and in equal amount to the C7VP control, while C7LBDC showed no detectable binding. In contrast, the fusion proteins C7LBDA and C7LBDB did not bind to the oligonucleotide containing only one C7 site, while C7VP bound only once, as expected. C7LBDA and C7LBDB bound equally to the oligo 1 and oligo 3, which contain two sites as inverted repeats with 3 intervening spaces or direct repeats with 9 intervening spaces, respectively. These data indicate that the ZFP-LBD fusion proteins dimerize and bind preferentially to DNA containing two C7 half sites, but that the exact orientation and spacing of the half sites is not critical. This flexibility in DNA binding site orientation may reflect the lack of a dimerization function in the C2H2 domains, but it is noteworthy that wild type estrogen receptor has also been shown to bind a variety of response

elements differing from the consensus ERE, including inverted and direct repeats.

To further confirm the homodimer binding stoichiometry of the ZFP-LBD fusion proteins and to demonstrate their DNA sequence specificity, the following experiment was conducted. A second ZFP-LBD fusion protein was constructed using the C2H2 zinc finger domain E2C(HS1), which binds to a recognition sequence 5'-GGG GCC GGA g 3' that differs in six out of nine base pairs from the C7 binding site. (Note that the lower case g denotes a 10<sup>th</sup> base that makes a minor contribution to the protein:DNA contact affinity.) Maps of specific examples constructed in the expression vector pcDNA3.1 are shown in FIG. 11 (E2CLBDAS) (SEQ ID NO: 11) and FIG. 12 (E2CLBDBS) (SEQ ID NO: 12).

Oligonucleotides were prepared containing an inverted repeat of two C7 sites, two E2C sites, or a mixed heterodimeric site of one C7 and one E2C half site. Two fusion proteins having different DNA binding domains (C7 or E2C) were tested for their DNA binding specificity against three oligonucleotides containing palindromic binding sites specific for C7, E2C or the combination of the two.

C7 oligo: gat cca aag tcg cgt ggg cgc agc gcc cac gcg atc aaa ga (SEQ ID NO: 51)

C7/E2C oligo: gat cca aag tcg cgt ggg cgc act ccg gcc ccg atc aaa ga (SEQ ID NO: 52)

E2C oligo: gat cca aag tcg ggg ccg gag act ccg gcc ccg atc aaa ga (SEQ ID NO: 53)

Gel shift assays were performed according to the standard protocol described above.

The results showed that C7LBD fusion protein only binds strongly to the oligonucleotide containing two C7 sites, but not to either the 2 x E2C probe or the C7/E2C probe. Likewise, the E2C-LBD chimeric protein only binds strongly to the 2 x E2C probe. Finally, neither ZFP-LBD construct binds to the oligonucleotide with the heterodimeric site. When

the two proteins were mixed in equal amount, a C7LBD and E2CLBD heterodimer was formed. The heterodimer binds to the heterodimeric probe. These results confirm that the ZFP-LBD fusion proteins are binding DNA preferentially as dimers. Furthermore, these data demonstrate good DNA binding specificity between fusion proteins with different C2H2 binding site preferences.

#### EXAMPLE 5

##### Ligand-dependent Regulation of Transgene Expression by ZFP-LBD Fusion Proteins

In order to evaluate the ability of the fusion proteins C7LBD A, B, and C to regulate transgene expression, a standard co-transfection reporter assay was performed. A reporter construct, henceforth known as 6x2C7pGL3Luc, containing six copies of a directly repeated C7 binding site (6x2C7) inserted upstream of an SV40 promoter fragment and reporter gene encoding firefly luciferase (pGL3Pro; Promega) was transfected along with the designated fusion protein and assayed as described below.

Cultured cells (HeLa, Cos, Hep3B or other) were seeded at  $5 \times 10^4$  cells/well in a 24 well plate prior to the day of transfection in DMEM Phenol-free media, supplemented with L-glutamine and 5% (v/v) charcoal-dextran stripped Fetal Bovine serum (sFBS). Cells were transfected using the Qiagen Superfect Transfection method. For each well 1  $\mu\text{g}$  of total DNA, containing 0.5  $\mu\text{g}$  luciferase reporter plasmid (6X2C7pGL3proLuc), 0.1  $\mu\text{g}$  of chimeric activator DNA (e.g., C7LBDA, C7LBDB, or C7LBDC) unless otherwise indicated, and 0.4  $\mu\text{g}$  of an inert carrier plasmid DNA (p3Kpn), was mixed with 60  $\mu\text{L}$  of DMEM phenol-free/serum free media, and 5  $\mu\text{L}$  of Superfect reagent. In general, about 10 ng to about 0.5  $\mu\text{g}$  of chimeric activator DNA was used for each well.

The mixture was vortexed for 10 seconds and incubated at room temperature for 10 minutes, followed by the addition of 350  $\mu\text{L}$  of DMEM phenol-free 5% sFBS media. Cells were washed once with Dulbecco's

phosphate buffered saline (DPBS) and the transfection mixture placed on the cells. Cells were washed once with DPBS following a 2.5 hour incubation at 37 degrees Celsius, and re-fed with DMEM Phenol-free 5% sFBS media.

At approximately 24 hours post-transfection, cells were treated with an inducing agent, 17  $\beta$ -Estradiol or 4 OH-Tamoxifen as indicated, each at 100nM final concentration in DMEM Phenol-free 5% sFBS. Cells were harvested 24 hours later by washing once with DPBS and adding 200  $\mu$ L 1X reporter lysis buffer (Promega). Plates were frozen at -80 ° C and thawed at room temperature for 1.5 hours on an orbital shaker at 100 RPM. After allowing for cellular debris to settle, lysate was diluted 1:10 with 1X reporter lysis buffer, and 10  $\mu$ L transferred to 96 well opaque plates. Plates were analyzed with a Tropix TR717 Microplate Luminometer using firefly luciferase substrate (Promega).

The ability of C7LBD short form chimeric proteins A, B, and C to regulate reporter gene expression in an estrogen-dependent manner was studied in Cos and HeLa cells. The constitutive activators C7VP16 and 2C7VP16 were used as positive controls. The results show that the three ZFP-LBD fusion proteins gave a similar profile in Cos and HeLa cells. All three ZFP-LBD fusion proteins had an estrogen dependent effect on the luciferase reporter gene. The characteristic pattern is that A has greater total activity than B and B has greater total activity than C. Likewise, the basal or ligand-independent effect of these proteins on the reporter gene follows a similar pattern; A > B > C. The estrogen dependent effect on gene expression ranged from two-fold to nine-fold in these experiments.

The regulation of luciferase reporter gene by the C7LBD long and short form fusion proteins was compared in Cos cells. The results indicate that the long form fusion proteins, which contain the estrogen receptor F region, have a higher basal and ligand-independent effect on the reporter gene than the short form. As a result, the long fusion proteins give lower fold induction. This result may be due to an

enhanced, but ligand-independent, transactivation activity in the F region that works synergistically with the heterologous VP minimal domain trimer. Alternatively, this result could be due to the difference in spacing, as a result of the intervening F region, between the VP activation domain and the estrogen receptor ligand binding domain of the recombinant proteins.

In order to evaluate the role of the composition of the heterologous transactivation domain on the activity of the C7LBD fusion proteins, the VP minimal domain trimer was replaced with either the carboxy terminal activation domain from human STAT-6 (amino acids 660 — 847) or the full length VP16 activation domain of approximately 77 amino acids (residues 413 — 490) (FIG. 13). In constructs with full length VP16, the transactivation domain was added either native, or in conjunction with an SV40 nuclear localization peptide sequence at the amino terminus of the VP16. C7LBD fusion proteins A or B containing different transactivation domains (TA2, STAT6C, VP16 and NLSVP16) were constructed and evaluated for their effects on gene activation and ligand induction. The construct, shown schematically and abbreviated above, includes the following:

1. C7ASTA2, C7BSTA2: C7LBD A or B short form with the VP16 minimal domain trimer.
2. C7BS-STAT: C7LBDB short form with the STAT6 carboxy activation domain.
3. C7BS VP16: C7LBDB short form with full length VP16 activation domain.
4. C7AS nlsVP16: C7LBDA short with full length VP16 preceded by a nls.

Assays were performed with Hela cells transfected with 0.5 ug of 6x2C7pGL3Luc reporter and 0.1  $\mu$ g regulator, Luc activity was determined as previously described. When the human STAT6 transactivation domain was used to replace the TA2 VP minimal domain

trimer, the same low basal activity and 9 fold ligand dependent induction of transgene, two-fold less than with the TA2 domain, was obtained.

The incorporation of NLS upstream of the full length VP16 (FIG. 24, C7ASnlsVP16) greatly increased the folding induction compared to TA2 or VP16 without the NLS, but the total activity was significantly decreased. When the full length VP16 domain was used, it gave about 2 fold higher total activity, but high basal activity resulting in weaker ligand dependent induction (3-fold).

#### EXAMPLE 6

##### **Ligand-Independent Activity of C7-PBD-VP16 Constructs Depends On The Structure of the Reporter Constructs**

In initial tests, the C7-PBD-VP16 construct showed the high basal (i.e. ligand-independent) activity. Thus, C7-PBD-VP16 was compared to the original, Gal4-based construct GL914VPc', which reportedly had a very low basal activity. When the GL914VPc' protein was tested on a 6xGal4-SV40 promoter-luciferase reporter, it displayed even higher basal activity than C7-PBD-VP16. Variation of effector/reporter ratios had no effect on the basal activities in both systems. It was discovered, however, that the ratios for optimal induction were different for GL914VPc' and C7-PBD-VP16, namely 1/30 and 1/10, respectively.

Other possible sources of ligand-independent activity were examined. Commercially available fetal calf serum (FCS) batches are known to contain estrogen or estrogen-like activities. Since it was possible that the presence of progesterone-agonistic activities in the serum was the cause for the high basal activities, the FCS was "stripped" of steroids using dextran-coated charcoal. However, side-by-side comparison of stripped and non-stripped serum showed no detectable difference in the basal activity of the switch constructs. Lipid-based transfection reagents such as Lipofectamine™ can also have significant agonistic activity on steroid receptors. Thus, the non-lipid transfection

reagent Superfect™ from Qiagen was used as an alternative, and compared to Lipofectamine™.

No reduction of the basal activities was observed. For all the assays described above, HeLa cells were used. However, the use of HepG2 cells, which were used in the original study with GL914VPc', brought no improvement.

The reporter p17x4TATA-luc, used in the original studies on Gal4, contains four Gal4 dimer binding sites upstream of a TATA box. GL914VPc' had a very low basal activity on this reporter, and was inducible by RU486. An equivalent reporter, pGL3TATA/10xC7, was therefore constructed to test C7-PBD-VP16. While the basal activity using a reporter construct having TATA reporter was still higher than in the Gal4 system, basal activity was clearly lower than using the SV40 promoter-containing pGL3prom/10xC7. Two additional reporters with minimal CMV promoters, pGL3minCMV/6xGal4 and pGL3minCMV/10xC7, were also constructed. The basal activity of the corresponding switch proteins was as high on these reporters as on the SV40 promoter containing reporters.

These results indicate that GL914VPc' and C7-PBD-VP16 were constitutively located in the nucleus and able to bind to their target sites, either as monomers or as dimers. However, unless bound to ligand the fusion proteins are only able to activate transcription in the context of more than a TATA box, i.e. a SV40 promoter or a minimal CMV promoter. If there is only a TATA box, ligand binding presumably associated with a conformational change is required for efficient activation of transcription.

It was found that ligand-independent basal activity is also cell type specific. C7-PBD-VP16 had an even lower basal activity on the TATA reporter in NIH/3T3 cells than it had in HeLa cells.

Since C7-PBD-VP16 appears to be constitutively translocated to the nucleus, the SV40 nuclear localization signal (NLS) between PBD and

VP16 domains was removed in the hope of making nuclear translocation more ligand dependent. The resulting construct, C7-PBD-VP16noNLS, was then tested on the pGL3prom/10xC7 reporter. However, transcriptional activation was no more RU486-dependent than in the case of C7-PBD-VP16 as shown by an unchanged basal activity. The construct C7-PBD $\Delta$ NLS-VP16noNLS was made in which the small remaining part of a natural SV40-like NLS at the N-terminus of the PBD (aa 640-644) is also removed.

### EXAMPLE 7

#### Optimizing Spacing and Orientation of the DNA Binding Domain Half-Sites

Naturally occurring steroid hormone receptors typically bind to an inverted repeat, or palindromic SRE. However, it has been shown in several cases that there is some flexibility in binding. Direct repeats and everted repeats can also serve as response elements. To determine the optimal spacing and orientation of the two half-sites for binding of a steroid receptor-based switch construct a total of eighteen C7 dimer TATA-luciferase reporter constructs were prepared. Six C7 dimers each in direct, inverted and everted repeat orientation, with spacers of 0 to 5 intervening bases. A test of the RU486-responsive C7-PBD-VP64 protein on each of these reporter constructs revealed that indeed there was quite some flexibility, since RU486 inducible activation was observed with each of the reporters (Tables 7-9, below; values listed are means of two determinations and the standard deviation). There were clear differences in the degree of responsiveness of each of the reporters.

A direct repeat of two C7 sites without any spacing displayed the most favorable properties. This is particularly important, indicating the ability to target (GNN)<sub>6</sub> sites using homodimeric and heterodimeric recombinant ligand-responsive transcription factors.

Further tests on the RU486-responsive VP64-C7-PR protein and the tamoxifen-responsive VP64-C7-ER protein, on each of these reporter constructs also revealed some flexibility, since ligand-inducible activation

was observed with each of the proteins on each of the reporters. However, the most favorable properties were observed with the VP64-C7-ER protein on the direct and everted repeats with a spacing of 3bp. Direct repeat with a spacing of 5bp was also more or less reasonable, permitting targeting of the *erbB-2* promoter with a 3 finger construct (see below).

Further studies have shown that binding of a C7/Cf2-PBD-VP64 heterodimer to a C7-Cf2 TATA reporter, with one binding site each for C7 and Cf2 without spacing, provides about a two-fold ligand-dependent change in transcription.

<b>Table 7</b>		
C7-PBD-VP64	Direct Repeats	
	Mean	STD DEV
C7c7	4081	511
C7c7 + RU486	20018	2090
C7ac7	3383	396
C7ac7 + RU486	8205	2064
C72ac7	3417	348
C72ac7 + RU486	8169	634
C73ac7	3269	1550
C73ac7 + RU486	5138	2319
C74ac7	3966	298
C74ac7 + RU486	6945	1377
C75ac7	2597	416
C75ac7 + RU486	5460	207

**TABLE 8**

C7-PBD-VP64	Inverted Repeats	
	Mean	STD DEV
<b>C77c</b>	2921	1368
<b>C77c + RU486</b>	10811	1596
<b>C7a7c</b>	4342	153
<b>C7a7c + RU486</b>	9534	2943
<b>C72a7c</b>	6964	573
<b>C72ac7 + RU486</b>	19186	3284
<b>C73a7c</b>	7132	5208
<b>C73a7c + RU486</b>	12844	171
<b>C74a7c</b>	3502	416
<b>C74a7c + RU486</b>	8855	2379
<b>C75a7c</b>	4704	105
<b>C75a7c + RU486</b>	12444	2117

**Table 9**

C7-PBD-VP64	Everted Repeats	
	Mean	STD Dev
<b>7cc7</b>	8750	1839
<b>7cc7 + RU486</b>	17377	1335
<b>7cac7</b>	6029	613
<b>7cac7 + RU486</b>	13599	2014
<b>7c2ac7</b>	7880	1720
<b>7c2ac7 + RU486</b>	20825	8197
<b>7c3ac7</b>	9670	1187
<b>7c3ac7 + RU486</b>	21491	274
<b>7c4ac7</b>	6974	441
<b>7c4ac7 + RU486</b>	8896	2455
<b>7c5ac7</b>	6892	388
<b>7c5ac7 + RU486</b>	13124	3490

### EXAMPLE 8

#### C7-PBD-Repressor Domain Fusion Constructs.

To evaluate the use of PBD fusion proteins as regulatable transcriptional repressors, C7-PBD was fused to a number of repressor domains (Table 10, below). When tested in luciferase reporter assays, many repressor constructs had no significant activity. C7-PBD-KK (containing a dimer of two KRAB-A boxes) reproducibly led to a 25-50% repression, which was largely RU486-dependent. A much stronger repression which, however, was largely RU486-independent was observed with a C7-PBD-SKD construct.

### EXAMPLE 9

#### Regulation Of *erbB-2* Promoter Activity With Three Finger-PBD-VP64 Homo-/Hetero-Dimers

The C7-PBD-VP16 switch protein was able to regulate 10xC7 reporter constructs, which contain 10 direct repeats of C7 sites with a spacing of 5bp (see above), indicating that a switch dimer can bind to direct repeats with this specific spacing. To evaluate the potential use of homo- and hetero-dimeric three finger-PBD fusion proteins for the ligand-dependent regulation of *erbB-2* promoter activity, the promoter region was screened for the presence of (GNN)<sub>3</sub>N<sub>5</sub>(GNN)<sub>3</sub> motifs. Four dimer target sites (E2E, E2F, E2G, and E2H) were identified. E2E overlaps with the 18bp E2C target sequence and could serve as a binding site for a homodimer. The other three sites have the potential to serve as heterodimer binding sites. The seven required three finger proteins were generated by F2 stitchery and analyzed for binding by ELISA (Table 11, below). *erbB-2*-specific switch constructs were then generated by fusion of each three finger protein to PBD-VP64, and tested for their ability to regulate *erbB-2* promoter activity. The values are mean and standard deviation of duplicate measurements. Only the heterodimeric E2F-PBD-VP64 switch led to a detectable regulation of the *erbB-2* promoter. This

regulation was not RU486-dependent, consistent with the high basal activities of C7-PBD-VP16 and C7-PBD--VP64 proteins.

**Table 10**  
**Progesterone Receptor Based Ligand-Responsive Transcription Factors**

	DNA Binding Domain	Ligand Binding Domain	Transcription Effector Domain
5	C7	hPR (aa 640-914)	VP16
	C7	hPR (aa 640-914)	VP64
	C7	hPR (aa 640-914)	KRABa
	C7	hPR (aa 640-914)	Mad
10	C7	hPR (aa 640-914)	Mad-Mad
	C7	hPR (aa 640-914)	KRABa-Mad
	C7	hPR (aa 640-914)	Mad-KRABa
	C7	hPR (aa 640-914)	Deactylase
	C7	hPR (aa 640-914)	SKD
15	2C7	hPR (aa 640-914)	VP16
	2C7	hPR (aa 640-914)	VP64
	E2E 3F	hPR (aa 640-914)	VP64
	E2F 3F	hPR (aa 640-914)	VP64
	E2G 3F	hPR (aa 640-914)	VP64
20	E2H 3F	hPR (aa 640-914)	VP64
	E2C(SP1) 6F	hPR (aa 640-914)	VP16
	E2C(SP1) 6F	hPR (aa 640-914)	VP64
	E2C(SP1) 6F	hPR (aa 640-914)	KRABa
	E2C(SP1) 6F	hPR (aa 640-914)	Mad
25	E2C(SP1) 6F	hPR (aa 640-914)	KRABa-KRABa
	E2C(SP1) 6F	hPR (aa 640-914)	Mad-Mad
	E2C(SP1) 6F	hPR (aa 640-914)	KRABa-Mad
	E2C(SP1) 6F	hPR (aa 640-914)	Mad-KRABa

**Table 11**

Target	Target Sequence	Binding	Mean Basal Activity	STD DEV Basal Activity	Mean RU486 Activity	STD DEV RU486 Activity
Control pcDNA 3.1			17209	1878		
E2C-HS1	ggg-gcc-gga	good				
E2C-HS2	gcc-gca-gtg	good				
E2E	gcc-gga-ggc	none	18259	140	15893	2083
E2F-HS1	gag-gag-ggc	good	61401	25291	54986	19240
E2F-HS2	gag-gaa-gta	?				
E2G-HS1	ggg-gcc-ggg	weak	25982	5444	12394	139
E2G-HS2	ggc-gca-gta	weak				
E2H-HS1	ggc-gcg-ggg	weak	15374	844	15374	537
E2H-HS2	ggg-gct-gcg	none				

**EXAMPLE 10****Estrogen And Progesterone Receptor Fusion Proteins With N-Terminal Effector Domains**

Recombinant ligand-responsive polypeptides were constructed using an estrogen receptor (ER) ligand binding domain (EBD). A Myc-ER fusion construct was obtained from Eliane Muller and used as a source of the EBD coding region. Rather than containing the human wild type amino acid sequence, Myc-ER contains a point mutation (aa 282-599, G525R) mouse EBD which has been shown to no longer bind estrogen, but bind the estrogen antagonist 4-OH tamoxifen, and paradoxically becomes activated by it. This has advantages for *in vivo* applications and for tissue culture experiments, not only because serum contains estrogen but also because phenol red present in all tissue culture media acts as an estrogen agonist.

The VP16-C7-ER, VP16-NLS-C7-ER, and VP16-C7-NLS-ER fusion constructs were prepared as described above. In parallel, an analogous set of progesterone receptor (PR) variants was also prepared (VP16-C7-PR, VP16-NLS-C7-PR, and VP16-C7-NLS-PR. The PBD in these

constructs encompasses aa 640-914 and therefore lacks the partial natural NLS (aa 640-644).

Each of these constructs was tested in a luciferase assay and compared to C7-PBD-VP16, using pGL3prom/10xC7 as a reporter. Not only did all these PR constructs have a higher activity in the presence of RU486 than C7-PBD-VP16, but the completely NLS-free VP16-C7-PR also had a significantly lower basal activity. This resulted in a dramatically improved ligand-dependent induction, 26-fold vs. 6-fold in this particular experiment. Tamoxifen-induced activity of the ER constructs was roughly four times higher than RU486-induced activity of the PR variants. Ligand-dependent induction was better; 43 fold for VP16-C7-ER.

The VP16 domain in VP16-C7-PR and VP16-C7-ER has been replaced by the following effector domains: the activator VP64, and the repressors KK (KRAB-A box dimer), MM (dimer of the Mad sin3 interaction domain) and SKD. The VP64 variants are useful, for example, in studies to determine the optimal spacing and orientation of the two half-sites, using the above-mentioned C7 dimer-TATA luciferase reporters

#### EXAMPLE 11

##### Targeting natural promoters using 3 Finger proteins fused to nuclear hormone LBDs

The following target sequences for 3 Finger switch homo- and hetero-dimers have been identified in the human *erbB-2* (E2) and integrin  $\beta 3$  (B3) promoters:

E2E	<u>GCC GGA GCC</u> ATGGG <u>GCC GGA GCC</u>	direct repeat,
5bp spacing homodimer (SEQ ID NO: 54)		
B3D	CGC TCC CTC TCA <u>GGC GCA GGG</u>	everted repeat,
3bp spacing, heterodimer (SEQ ID NO: 55)		

B3E                    GGC GCC CAC TGT GGG GCG GGC                    everted repeat,  
3bp spacing, heterodimer (SEQ ID NO: 56).

## EXAMPLE 12

### Targeting Natural Promoters Using Six Finger Proteins Fused To Nuclear Hormone Ligand Binding Domains

#### *The "6 Finger heterodimer"*

Regulation of a 6 finger protein binding to a single 18bp site using any of the formats described have been unsuccessful. Similarly, a C7-PBD-VP64 protein did not activate a TATA reporter containing only a single C7 site. As an alternative, heterodimer constructs were prepared in which only one of the dimerization partners contains a DNA binding domain, while the other contains an effector domain.

The formats were as follows:

- (1) E2C-PR // PR-VP64
- (2) E2C-ER // ER-VP64

All four fusion constructs were fully sequenced and tested in a luciferase assay for their ability to regulate the *erbB-2* promoter in a ligand-dependent manner. It was found that the PR 6 Finger heterodimer was inactive; a similar observation was made with an C7-RxR // EcR-VP16 heterodimer. In contrast, the E2C-ER // ER-VP64 heterodimer had some activity, and the addition of Tamoxifen lead to a roughly three-fold upregulation of promoter activity. Variations in the ratio of the two heterodimerization partners led to an increased inducibility, up to total of 5.3-fold.

The coding region for RXR (mammalian) and EcR (*Drosophila*) were PCR amplified from pVgRXR (Invitrogen) using the primers listed below and AmpliTaq DNA Polymerase (Hoffmann-LaRoche). Forward and backward primers were chosen to allow construction of the constructs. The cycling conditions were 2'/94° C C; 25 x (30''/94° C — 30''/60° C — 2'/72° C); 10'/72° C. The PCR product was purified with the Quiagen PCR prep kit, cut with the indicated restriction endonucleases and ligated

into a modified eukaryotic expression vector pcDNA3 (Invitrogen; see, also, Beerli *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:14628-14633) to yield the constructs in FIG. 14.

Primers:

(FseI)-RXR: (SEQ ID NO: 57)

GAGGAGGAGGGCCGCGCCGGAAGCCGTGCAGGAGGAGCGGC

RXR-(AseI): (SEQ ID NO: 58)

GAGGAGGAGGGCGCGCCAGTCATTGGTGCGGCGCCTCCAGC

RXR-(PacI): (SEQ ID NO: 59)

GAGGAGGAGTTAATTAAAGTCATTGGTGCGGCGCCTCCAGC

(FseI)-EcR: (SEQ ID NO: 60)

GAGGAGGAGGGCCGCGCGGGTGGCGGCCAAGACTTTGTTAAGAAGG

(SfiI)-EcR: (SEQ ID NO: 61)

GAGGAGGAGGGCCAGGCGCGCGGTGGCGGCCAAGACTTTGTTAAGAA  
GG

EcR-(AseI): (SEQ ID NO: 62)

GAGGAGGAGGGCGCGCCCGCATGAACGTCCCAGATCTCCTCGAG

*Exchange of zinc finger and effector domains*

After digestion with the restriction endonuclease SfiI the C7 3-finger protein was replaced with the 6-finger proteins E2C, B3B, B3C2 and 2C7 by standard cloning procedures. After digestion with the restriction endonucleases AseI and PacI the activation domain VP16 was replaced with the activation domain VP64 and the repression domains KK and SKD.

*Luciferase assays*

For all transfections, HeLa cells were plated in 24-well dishes and used at a confluency of 40-60%. Typically, 175 ng reporter plasmid (pGL3-promotor constructs or, as negative control, pGL3basic) and 25 ng effector plasmid (zinc finger constructs in pcDNA3 or, as negative control, empty pcDNA3.1) were transfected using the Lipofectamine reagent (Gibco BRL). Cell extracts were prepared approximately 48 hours

after transfection. Luciferase activity was measured with the Promega luciferase assay reagent in a MicroLumat LB96P luminometer (EG&G Berthold).

#### *Bombyx mori* EcR

A plasmid (LNCVBE) containing the coding region for *Bombyx mori* EcR was obtained from F. Gage. *Bombyx mori* EcR is PCR amplified from this plasmid using the primers listed below and AmpliTaq DNA Polymerase (Hoffmann-LaRoche). Forward and backward primers were chosen to allow construction of the constructs corresponding to FIG. 14 but replacing *Drosophila* EcR by *Bombyx mori* EcR.

(FseI)-BE: (SEQ ID NO: 63)

GAGGAGGAGGGCCGGCCGAGGCCTGAATGTGTCATACAGGAGCCC

(SfiI)-BE: (SEQ ID NO: 64)

GAGGAGGAGGGCCAGGCCGAGGCCTGAATGTGTCATACAGGAGCCC

BE-(AscI): (SEQ ID NO: 65)

GAGGAGGAGGGCGCGCCCTCCGCCACGTCCCAGATCTCCTCGAG

#### **C7-R-VP16 // C7-E-VP16**

This heterodimer was examined on two reporters, one containing 10 C7 sites and one containing 6 C7 sites, and in two cell lines, HeLa and NIH. In all cases the C7-R-VP16 construct alone showed a high activation of transcription (840-fold) that did not depend on the presence of Ponasterone A. However the C7-E-VP16 construct showed a very little activation of transcription on its own. C7-R-VP16 // C7-E-VP16 together showed the same behavior as C7-R-VP16 alone.

#### **C7-R // E-VP16**

In this heterodimer, the activation domain on RXR is dropped to eliminate the basal activation observed above. EcR has no DNA-binding domain to render activation dependent on the presence of DNA-bound RXR. This heterodimer was tested with the 3-finger protein C7 on the 10C7 reporter and with the 6-finger protein E2C on the E2P reporter that

contains a single E2P binding site. In both cases no significant activation could be observed.

#### **C7-R // C7-E-VP16**

To combine the low basal activity of C7-R // E-VP16 with the high activation seen with C7-R-VP16 // C7-E-VP16, the activation domain on RXR was dropped but the zinc finger protein on EcR was retained. In this set-up, on a 6x2C7 reporter, a 5-fold activation with very low basal activity was observed. Similar constructs using the more powerful VP64 activation domain have also been made.

#### **E2C- ER // ER-VP64**

This heterodimeric onstruct showed 5.3 fold tamoxifen-dependent activation at ratios of 6.7/60 and 2.2/60 of the erbB-2 promoter.

#### **E2C- ER // ER-KRAB**

This heterodimeric construct showed 2.9 fold tamoxifen-dependent repression of the erbB-2 promoter at a ratio of 1/10.

#### **B3B/B3C2-ER // ER-VP64**

This six finger heterodimeric construct showed 4.5 - 7.8 fold tamoxifen-dependent activation of the  $\beta 3$  promoter.

### **EXAMPLE 13**

#### **Regulation Of Endogenous ErbB-2 Gene Expression Using Adenovirus-Mediated Delivery Of E2C-KRAB**

Adenovirus vectors can be produced at very high titers, which makes them useful for gene therapy applications. To demonstrate the use of the E2C-KRAB repressor protein in animal models, E2C-KRAB (and, as a control, 2C7-KRAB) encoding adenoviruses were generated. The method for adenovirus production is described in detail, for example, in He *et al.* (1998) *Proc. Natl. Acad. Sci. U.S.A.* 95:2509-2514.

Briefly, the zinc finger coding regions were excised from the pMX/E2C-KRAB and pMX/2C7-KRAB bicistronic retrovirus plasmids by BamH1-Not1 digest. The resulting fragments were then subcloned into the Bgl2-Not1 sites of pAdTrack-CMV. After linearization with Pme1,

pAdTrack plasmids were co-electroporated with circular pAdEasy-1 into BJ5183 cells. This bacterial strain is not *recA* and therefore allows homologous recombination between the 2 plasmids. Electroporated cells were then plated onto Kan plates. Only plasmids that have recombined together provide Kanamycin resistance, because this marker is only present on pAdTrack. After screening to distinguish recombinants from background (due to incomplete linearization of pAdTrack plasmids), the linear adenovirus vector genomes were released from the recombinant pAdEasy/E2C-KRAB and pAdEasy/2C7-KRAB plasmids by Pac1 digest.

The linearized vectors were then transfected into 293 cells. This cell line makes the Adeno E1A and E1B proteins, which have been deleted from the vector and are required for replication.

#### EXAMPLE 14

##### **Modifications To The Estrogen Receptor Ligand Binding Domain Improve Ligand Dependent Induction And Ligand Selectivity.**

Single amino acid mutations in the estrogen receptor ligand binding domain can have a significant effect on the basal and ligand dependent level of gene activation. For example, a glycine to valine substitution at estrogen receptor residue 400, has been described as a destabilizing or temperature sensitive mutation (White (1997) *Adv. Pharmacol.* 40:339-367; Aumais *et al.* (1996) *J. Biol. Chem.* 272:12229-12235). The effect of this mutation on the properties of the fusion proteins was tested. The general methods for constructing fusion proteins with altered amino acids is described below.

Mutagenesis of the fusion proteins C7LBDA and C7LBDB was performed using oligonucleotide mediated site directed mutagenesis (Stratagene; Quikchange Site-Directed Mutagenesis Kit) to either substitute arginine for glycine at amino acid 521 (G521R-human estrogen receptor nomenclature) or a valine residue for glycine at amino acid 400 (G400V). The sequences of the oligonucleotides used for G521R mutagenesis were

GTACAGATGCTCCATGCGTTTGTTACTCATGTGCC (SEQ ID NO: 66) for the noncoding strand and GGCACATGAGTAACAAACGCATGGAGCATCTGTAC (SEQ ID NO: 67) for the coding strand, where the nucleotide in bold represents the change from the wild type sequence.

The sequences of the oligonucleotides used for G400V mutagenesis were CCATGGAGCACCCAGTGAAGCTACTGTTTGC (SEQ ID NO: 68) for the coding strand, and GCAAACAGTAGCTTCACTGGGTGCTCCATGG (SEQ ID NO: 69) for the noncoding strand, where the nucleotide in bold represents the change in sequence from wild type.

Templates were added at 10 ng to 50 ng per reaction with 125 ng of each primer in 10mM KCl, 10mM  $(\text{NH}_4)_2\text{SO}_4$ , 20mM Tris-HCl (pH 8.8), 2mM  $\text{MgSO}_4$ , 0.1% Triton X-100, 0.1 mg/ml BSA, dNTP mix, and 2.5U PfuTurbo™ DNA polymerase. The reactions were carried out on a Perkin Elmer GeneAmp PCR system 9600 thermal-cycle using an initial temperature of 94 degrees Celsius for 30 seconds to denature the template, followed by 12 cycles at 95 degrees Celsius for 30 seconds, 55 degrees Celsius for 1 minute, and 68 degrees Celsius for 4 minutes, with a single round of extension at 72 degrees Celsius for 2.5 minutes. PCR samples were treated with 10U *DpnI* for 1hr at 37 degrees Celsius to digest the non-mutagenized parent template.

DH5 $\alpha$  supercompetent Epicurean Coli® XL-1 cells were transformed by combining 1  $\mu\text{L}$  of the *DpnI* treated PCR samples with 50  $\mu\text{L}$  of the cells in chilled Falcon 2059 tubes, incubated on ice for 30 minutes, heat shocked at 42 degrees Celsius for 45 seconds and chilled on ice for 2 minutes. A 500  $\mu\text{L}$  aliquot of SOC media pre-warmed to 42 degrees Celsius was added to the transformation reaction and incubated for 1 hour at 37 degrees Celsius with shaking. The transformed cells were plated onto LB plates containing 100  $\mu\text{g/ml}$  ampicillin and incubated for at least 16 hours.

Mutation efficiency was determined by altering a nonsense codon in a  $\beta$ -galactosidase expression plasmid to glutamine and determining expression of  $\beta$ -galactosidase, as evidenced by IPTG/X-Gal plates. Approximately three clones for each mutation were selected for restriction enzyme digestion to check for template integrity, followed by dideoxynucleotide sequencing of the entire coding frame to confirm the desired mutation.

C7LBD (short) chimeric regulators A, B, and C with and without the G400V mutation in the estrogen receptor LBD were compared for their ability to induce expression of the 6x2C7pGL3Luc. As observed previously, the total activity of the three fusion proteins has the relationship A>B>C; this relationship was maintained with and without the G400V mutation. The pattern of basal expression was dramatically altered by the G400V mutation. The basal or ligand independent effect of the three C7LBD regulators with the G400V mutation is reduced to nearly the level of the reporter plasmid alone. As a result, the fold ligand dependent induction dramatically increases, for example from 10 fold to 420 fold for C7LBDA.

It has previously been observed with fusion proteins containing an estrogen receptor ligand binding domain, that activity could be induced by use of not only the natural agonist estrogen (E2) but also synthetic anti-estrogens such as 4-OH tamoxifen (Littlewood *et al.* (1995) *Nucl. Acids Res.* 23:1686-1690; Danielian *et al.* (1993) *Mol. Endocrinol.* 7:234-240). The ability of the C7LBD fusion to be induced by 4-OH-tamoxifen was demonstrated.

The results of the study showed the ligand-dependent regulation of a luciferase reporter gene construct in HeLa cells using three recombinant molecular constructs, C7LBDAS, C7LBDBS, C7LBDCS with and without a G400V mutation in response to estrogen (E2) and 4 hydroxytamoxifen (OHT). In particular, the results showed that fusion proteins C7LBD B and C are induced equally well by 100 nM tamoxifen or estrogen. For

C7LBDA, tamoxifen appears to be approximately two-fold more active than estrogen itself.

Another mutation of interest in estrogen receptor LBD is a glycine to arginine substitution at amino acid 521 of human estrogen receptor. This mutation has also been described in the mouse estrogen receptor homolog at the equivalent site of residue 525. This mutation ablates responsiveness of the mutated LBD to estrogen, but still allows the binding of the anti-estrogen tamoxifen (Littlewood *et al.* (1995) *Nucl. Acids Res.* 23: 1686-1690; Danielian *et al.* (1993) *Mol. Endocrinol.* 7:234-240). The effect of the G521R mutation on the activity of the C7LBD regulators was tested. C7LBDB was compared to C7LBDB (G400V) and C7LBDB (G521R).

The results of the study showed the ligand-dependent regulation of a luciferase reporter gene construct in HeLa cells using three recombinant molecular constructs: C7LBDBS, C7LBDBS with a G521R mutation and C7LBDBS with a G400V mutation in response to estrogen (E2) and 4-hydroxytamoxifen (OHT). Similar to the effect observed with the G400V mutation, G521R significantly reduces the basal activity of the fusion protein regulator. But most importantly, now the C7LBDB(G521R) regulator is fully activated by 100 nM 4-OH-tamoxifen, but completely inactive in response to estrogen. Note that the G400V mutant is still fully activated by estrogen and tamoxifen.

To further investigate the effect of the G521R mutation, a series of different estrogenic compounds were evaluated for their ability to induce the C7LBD regulators. The activity of 100 nM for four compounds: estrogen (E2) and diethyl-stilbesterol (DES) are estrogenic agonists, 4-OH-tamoxifen and raloxifen (Ral) are non-steroidal anti-estrogens, or so-called SERMS (selective estrogen receptor modulators) were compared.

The study tested ligand-dependent regulation of a luciferase reporter gene construct in Hep3BL liver cells using recombinant molecular constructs C7LBDBS with a G521R mutation and C7LBDBS with a G400V

mutation in response to estrogen (E2) diethylstilbesterol (DES), 4-hydroxytamoxifen (4-OHT) and raloxifen (Ralox). The results showed that the G521R mutation selectively eliminates response to the agonists, but the non-steroidal synthetic ligands tamoxifen and raloxifen are still fully active.

#### EXAMPLE 15

##### **Effect Of The Minimal Promoter Composition On Regulation Of Transgenes By ZFP-LBD Fusion Proteins**

The composition of the minimal promoter used in reporter assays can dramatically effect the level of gene expression. Likewise, the activity of natural steroid receptors varies on different gene targets depending on the composition of their promoters. Reporter constructs containing 6x2C7 binding sites upstream of a minimal TATA box promoter fragment derived from the c-fos gene, referred to here as TATA were constructed to show the effect on the level of regulation. C7LBD A and B fusions without or with the G400V or G521R mutations were compared. As observed previously on the pGL3 SV40 promoter, the G400V and G521R mutations significantly decrease the basal activity of the chimeras compared to those without these mutations. Further, the G521R mutant is selectively activated by tamoxifen. On this weaker minimal promoter, estrogen is only a weak inducer, while 4-OH-tamoxifen is significantly better. This effect is even more pronounced on C7LBD A compared to B; on chimera C7LBDA (G400V), tamoxifen is at least 10 fold more active than estrogen.

An experiment was done to directly compare the relative activity of the C7LBD chimeras on reporter constructs containing the stronger pGL3 SV40 promoter or the weaker c-fos TATA box promoter.

The results of the study show that the ligand-dependent regulation of a luciferase reporter gene construct expressed from a minimal TATA promoter in Hep3BL liver cells using recombinant molecular constructs C7LBDAS and C7LBDBS with a G400V mutation and C7LBDBS with a

G521R mutation in response to estrogen (E2) and 4-hydroxytamoxifen (OHT). Three important observations can be made: 1) the absolute level of induced activity is about 10 fold higher on the SV40 than the TATA promoter 2) the basal activity of the fusions is also about 10 fold higher on the SV40 than on the TATA promoter, 3) while both promoters show strong fold induction by tamoxifen (492 X on SV40 and 132 X on TATA), estrogen is only a strong inducer of the SV40 but not the TATA promoter (177X vs 14X). These results indicate that a gene regulation system using these fusion proteins can be "tuned" by choice of an appropriate minimal promoter.

*Target Selectivity of Different C2H2 DNA binding domains*

Reporter constructs with 3 copies of direct repeats of the C7 binding site (GCG TGG GCG) or E2C binding site (GGG GCC GGA g) inserted upstream of the promoter region in pGL3Luc were used to evaluate target specificity two different ZFP-LBDBs fusion protein regulators. ZFP-LBDB short fusions were constructed containing either the C7 DNA binding domain or the E2C DNA binding domain and tested on the two different reporter constructs. The study was designed to show the effect of three direct repeats of either C7 or E2C binding sites inserted upstream of the promoter of a luciferase reporter gene construct in HeLa cells on estrogen-dependent gene expression using recombinant molecular constructs C7LBDBS and E2CLBDBS. Estrogen-dependent induction only occurs when the chimera's DNA binding domain (DBD) matches the binding sites in the reporter. The E2CLBD chimera shows no increase of luciferase activity on the 3x2C7 Luc reporter and visa versa for C7LBD on the 3xE2C reporter.

It was previously determined from DNA binding studies that the fusion protein regulators have an absolute dependence on the presence of two half sites within a "response element" in order to bind DNA. In order to determine the optimal orientation and spacing of the binding sites for gene activation, a series of different reporter constructs were assembled.

In order to determine the optimal target DNA spacing and orientation of the C2H2 binding sites for transgene induction, C7LBDBS was transfected into HeLa cells and assayed for basal and tamoxifen induced activity on a series of reporter constructs.

A series of different reporter constructs assembled in order to determine the optimal target DNA spacing and orientation of the C2H2 binding sites for transgene induction, C7LBDBS was transfected into HeLa cells and assayed for basal and tamoxifen induced activity on a series of reporter constructs diagramed above. Reporter constructs were constructed by cloning double stranded oligonucleotides containing the various binding sites into the multiple cloning site of the pGL3Luc reporter. "Response elements" composed of direct, inverted (palindromic), and everted repeats of two C7 binding sites were compared; each response element was separated by two (2) bp except in the control 6 X 2C7, where spacing was 5 bp. Several arrays of directly repeated single C7 sites were tested with various spacing. The data show that direct repeats and everted repeats are preferred over palindromic binding sites. Further, 6 C7 sites, each separated by 2 bp is comparable to the control element of 6 x 2C7, even though it contains only half the number of individual C7 binding sites.

#### EXAMPLE 16

##### **Construction and Evaluation of ZFP-LBD Fusion Protein Regulators Containing Arrays of Six C2H2 Zinc Fingers**

Studies were performed to determine if DNA binding domains comprised of zinc finger arrays binding up to 18 bp of DNA could be substituted for the normal estrogen receptor DBD. The previous constructs, containing three finger arrays that bind nine bp are a fairly conservative replacement of the wild type estrogen receptor ligand binding domain that binds six bp for each receptor monomer. The possibility exists that if large DNA binding domains are fused to an LBD fragment, that these domains may prevent dimerization via the LBD

dimerization domain due to steric interference. However, since the six finger arrays already provide high DNA specificity and affinity, dimerization may be unnecessary for the DNA binding and activity of these fusions proteins. Fusion protein regulators were prepared by fusing the 2C7 six finger array to the three LBD fragments A, B, and C described above. FIG. 15 provides a schematic and description of the cloning step required to assemble 2C7LBDshort A, B, and C.

Protein binding to DNA was analyzed by gel shift assay. The electrophoretic studies used 2C7 recombinant molecular constructs using native PAGE and SDS PAGE analysis of binding to a DNA probe containing six 2C7 binding sites. In this experiment, the 2C7VP16 protein was used as a control and the P32-labeled DNA probe was the 6x2C7 fragment excised from the 6X2C7pGL3Luc. Sufficient 2C7VP protein was added to yield three distinct gel shifted products. When a similar level of protein for the 2C7LBD A, B, and C were applied, only a single weak band was observed. By comparison to the one and two copies bound bands for the 2C7VP16 control, the 2C7LBD band position suggests it is binding as a monomer. Furthermore, the weak level of binding compared to the 2C7VP16 control suggests the DNA binding affinity of the 2C7 domain is significantly reduced in the context of the LBD fusion protein. Results of *in vitro* expressed proteins by SDS-PAGE, indicated equal amounts of proteins expressed and the expected relative increase in size for the LBD A, B, and C forms.

The ability of the 2C7LBD A, B, and C fusion protein chimeric regulators to activate expression of the 6X2C7Luc reporter gene were evaluated essentially as described previously for the C7LBD studies. The results of the study show the ligand-dependent regulation of a 2C7 SV40 luciferase reporter gene construct in Cos cells using three recombinant molecular constructs, 2C7LBDAS (SEQ ID NO: 1), 2C7LBDBS (SEQ ID NO: 2), 2C7LBDCS (SEQ ID NO: 3), and a positive control, 2C7-Vp16. The results are similar to the data evaluating C7LBD in Cos cells. The

2C7LBD regulators give about two fold estrogen dependent induction over basal, with 2C7LBDA > B > C for both the total activation activity and the increased basal activity relative to reporter plasmid alone. Maps of the additional constructs are depicted in FIG. 16 - FIG. 22.

## EXAMPLE 17

### Construction and Evaluation of Additional Reporter Transgene Constructs

An inducible promoter was constructed based on binding sites for the 3 Finger protein N1. The promoter contains 5 direct repeats of N1 sites spaced by 3bp; the spacing between the 5 repeats is 6bp. (FIG. 23A)

Luciferase assay. HeLa cells were cotransfected with plasmids encoding the indicated fusion proteins and the N1 reporter construct. At 24h later, the cells were treated with 10nM RU486 (FIG. 23B) or 100nM Tamoxifen (FIG. 23C), respectively. At 48h post transfection, cell extracts were assayed for luciferase activity.

Another inducible promoter based on binding sites for the 3 Finger protein B3. The promoter contains 5 direct repeats of B3 sites spaced by 3bp; the spacing between the 5 repeats is 6bp (FIG. 24A).

Luciferase assay. HeLa cells were cotransfected with plasmids encoding the indicated fusion proteins and the B3 reporter construct. At 24h later, the cells were treated with 10nM RU486 (FIG. 24B), or 100nM Tamoxifen (FIG. 24C), respectively. At 48 h post transfection, cell extracts were assayed for luciferase activity.

## EXAMPLE 18

### Heterodimer Formation in Presence of Ligand

FIG. 25 shows the results of a luciferase assay showing RU486-induced formation of functional VP64-C7-PR/VP64-CF2-PR heterodimers. HeLa cells were cotransfected with the corresponding effector plasmids and TATA reporter plasmids (C7/CF2-dr0, C7 site 5' to a CF2 site, direct "repeat", no spacing; C7/C7-dr0, 2 C7 sites, direct repeat, no spacing).

At 24h later, the cells were treated with 10nM RU486. At 48h post transfection, cell extracts were assayed for luciferase activity.

#### EXAMPLE 19

##### **Construction and Evaluation of the Cys<sub>2</sub>-His<sub>2</sub> Zinc finger DBD – ER LBD regulators in Adenoviral Vectors**

In order to efficiently deliver the two components of the regulatory system to mammalian cells, either *ex vivo* or *in vivo*, a series of adenoviral vectors were constructed. These vectors contained either the ZFP-LBD fusion protein regulator linked to the immediate early CMV promoter or the regulatable transgene, linked to the 6 x 2C7 array of C7 binding sites and the minimal promoter from SV40 or c-fos TATA as described previously. The fusion protein regulator vector and regulatable transgene vector are then be mixed at various ratios and delivered to cells or animals by standard methods.

Construction of an adenovirus vector is routine and generally, the procedure involves three main steps: first a shuttle plasmid containing the viral left ITR, viral packaging signal, a promoter element, a transgene of interest linked to the promoter element and followed by a poly adenylation sequence, and some additional DNA sequences, viral or non-viral, required for recombination is prepared. Second, this left end shuttle plasmid, along with the remainder of the viral genome (i.e. the right end of the vector) are transfected into a host cell and joined through DNA recombination to form a complete vector genome. This recombination step may result from sequence homology between the two vector halves or may be aided by the use of site specific recombinases such as Cre and their corresponding LoxP recombination sequences. Finally, the newly formed virus is amplified up and purified in a series of steps. The details of the construction of these vectors are briefly described below.

##### **Left end shuttle plasmid construction for ZFP-LBD Fusion Protein Regulators**

Shuttle plasmids containing the left viral ITR, CMV immediate early promoter and ZFP-LBD regulator were prepared in the plasmid pAvCIX

(Figure 26). Note that this vector contains a loxP recombination site just downstream of the poly adenylation sequence. DNA encoding the intact reading frame for the chimeric regulators C7LBD As(G521R), C7LBD Bs(G521R), and C7LBD Bs(G400V) were excised from the appropriate pCDNA constructions, (see figures 4 and 5 for LBD As and LBD Bs constructs respectively) by digestion with restriction enzymes EcoRI and Not I. The ZFP-LBD DNA fragments were modified with Klenow to fill in the restriction site overhangs and blunt end ligated into the EcoRV at bp 1393 site of pAvCvI $\alpha$  to generate pAvCv-C7LBD As(G521R), pAvCv-C7LBD Bs(G521R), and pAvCv-C7LBD Bs(G400V).

#### **Construction of Left end shuttle plasmids containing regulatable transgene cassettes**

Two regulatable transgene cassettes were prepared. One contained the 6x2C7 binding sites and SV40 minimal promoter fragment linked to the Luciferase transgene as in pGL3 6x2C7-Luc (described in example 5). The second vector contained the 6x2C7 binding sites and c-fos TATA minimal promoter linked to a cDNA encoding murine endostatin fused to an amino terminal secretion signal. The complete sequence of this fusion protein is listed in SEQ ID NOs. 70 and 71.

These vectors were constructed in two steps. First, a fragment containing the CMV promoter and tri-partite leader sequence (TPL) of pAvCvI $\alpha$  (Fig. 26) was excised by digestion with MluI and BglII, which cut at bp 473 and 1375 respectively. The restriction site overhangs were filled in with Klenow. Blunt ended DNA fragments containing the 6x2C7-SV40 or 6x2C7-TATA enhancer/promoter regions of the previously described reporter plasmids were ligated into this backbone to create pAV-6x2C7SV40 and pAV-6x2C7TATA shuttle plasmids. Next, DNA fragments containing the Luciferase or murine endostatin transgenes were ligated into the EcoRV site of the appropriate shuttle plasmids to create pAV6x2C7SV40-Luc (lox) or pAV6x2C7TATA-mEndo (lox).

#### **Construction of a Right end vector plasmid**

To complete the vector construction, a plasmid containing the remainder of the viral vector genome is required. This plasmid, referred to as pSQ3, which is shown in Fig. 27, contains a pBR322-derived backbone, ampicillin resistance gene and the adenovirus serotype 5 genome, beginning at Ad5 bp 3329, through the right ITR, with deletions in the E2a and E3 region as described previously (Gorziglia *et al.* (1996) *J. Virol.* 70:4173-4178). In addition, this plasmid has two important features, a loxP site inserted at the Bam HI site (bp 31569) just upstream of the Ad5 sequences, and a Cla I site at the end of the viral 5' ITR. This Cla I site is used to linearize the plasmid and expose the right ITR during vector construction.

#### Vector Assembly and propagation

Three adenoviral vectors encoding fusion protein regulators, Av3CV-C7LBDAS(G521R), Av3CV-C7LBDBS(G521R), and Av3CV-C7LBDBS(G400V) and two vectors containing regulatable transgenes, Av3SV-LUC and Av3TATA-Endo were constructed. Each vector was generated by a standard procedure. Briefly, for each vector construct, three plasmids, pSQ3 (pre digested with ClaI), the appropriate left end shuttle plasmid (e.g. pAvCv-C7LBD As(G521R), or pAv6X2C7SV40-Luc (lox), pre-digested with NotI and Afl II, and an expression plasmid for the Cre recombinase, pCMV-CRE, were cotransfected at a weight ratio of 3:1:1 into dexamethasone induced AE1-2a cells (Gorziglia et al.) using Promega's Profection Kit. About 1 week after transfection, cells were harvested and lysed by 4 cycles of freeze/thaw. The resulting cell lysate was passed onto fresh dexamethasone induced AE1-2a cells and the culture maintained about a week until cytopathic effect (CPE) was observed. This process was repeated several cycles until sufficient material was obtained to purify the vector by CsCl equilibrium density centrifugation. Once purified, vectors are quantitated by lysing in buffer containing 10mM Tris, 1mM EDTA, 0.1% SDS for 15 minutes at 56°C, cooling and reading the absorbance at 260 nm wavelength (OD260).

The OD260 reading is converted to a virus particle concentration using 1 OD260 unit =  $1.1 \times 10^{12}$  particles/ml.

## Results

### In Vitro Regulation with Adenovirus Vectors

5 The ability to regulate expression of a transgene delivered by an adenovirus vector was demonstrated by the following experiment. HeLa cells were infected with a mixture of two adenovirus vectors, one containing a fusion protein regulator either (Av3-C7LBD-A(G521R) or Av3-C7LBD-B(G52R), the other containing the 6x2C7SV40-luc cassette. 10 To determine the optimal ratio of target vector to effector vector, two different doses of the transgene or target vector (50 or 250 viral particles per cell) at three different ratios of effector vector ( 50, 250, 750 particles per cell for each target dose) were tested. Twenty four hours after vector transduction, the cells were treated where appropriate with 100 nM 4-OH-tamoxifen. Following an additional 24 hrs incubation, the cells were lysed and assayed for luciferase activity. For the Av3CV- 15 C7LBD A(G521R) vector, the data indicat  relatively low levels of luc expression in the absence of 4-OHT, a strong 4-OHT dependent induction and a dose dependent increase in luc activity as more fusion protein regulator vector is used. At the highest doses (750 particles per cell) of 20 chimeric regulator vector tested, tamoxifen-specific induction of 460 to 560 fold over basal was achieved at target vector doses of 250 and 50 particles per cell, respectively.

25 The same experiment carried out using the LBD B version of the chimeric regulator; Av3CV-C7LBD B(G521R). For this vector, the fold induction and absolute luciferase activity were about two fold lower than obtained with the As-based regulator. These results are consistent with all the previous transient transfection experiments performed with plasmids. Notably, a first generation of Av3-chimeric regulator vectors 30 constructed with the RSV promoter driving the expression of the C7LBD gene did not yield good transgene upregulation of the Av3SV40-Luc

vector. Apparently, the expression level from the weaker RSV promoter was not adequate to produce the necessary levels of fusion protein.

### **In Vivo Regulation with Adenovirus Vectors**

To demonstrate the effectiveness of the C7LBD regulators to control transgene expression *in vivo*, a study was designed to evaluate three important variables: 1) the effectiveness of regulators containing either the G400V or G521R mutations, 2) the ratio of target and effector vector, and 3) the dose of 4-OHT. The importance of the G400V and G521R mutations are as follows. While the G521R mutation is selectively responsive to 4-OHT and is not affected by endogenous estrogen, it requires about a 10-fold higher drug concentration than the G400V mutation to achieve maximum activity. While the G400V is active at a lower dose of 4-OHT, it is also subject to induction by estrogen and could show higher basal activity *in vivo*.

Details of the animal study are as follow. On study day 1, C57Bl/6 male mice were given a total adenovirus vector dose of  $2 \times 10^{11}$  particles via tail vein injection. On day two blood samples were collected, then animals were injected i.p. with 200 ul of sunflower seed oil containing 5% DMSO and either no, 50 ug, or 500 ug of tamoxifen (Sigma # T56448). Blood samples were collected daily for three days following drug administration, and on study days 8 and 10. At the completion of the study, murine endostatin levels were determined by ELISA (Accucyte Kit, Cytimmune Sciences, Maryland).

The study groups included the following:

Negative Control –  $2 \times 10^{11}$  particles Av3Null, Ad vector with no transgene

Positive Control –  $2 \times 10^{11}$  particles Av3RSV-mEndo, constitutively expresses endostatin from the RSV promoter.

1:1 As521 – Received  $1 \times 10^{11}$  particles of Av3TATA-mEndo and  $1 \times 10^{11}$  particles of Av3Cv-C7LBDA(G521R); no treatment (basal) or + 50  $\mu$ g tamoxifen.

1:1 Bs400 – Received  $1 \times 10^{11}$  particles of Av3TATA-mEndo and  $1 \times 10^{11}$  particles of Av3Cv-C7LBDBs(G400V); no treatment (basal) or + 50  $\mu$ g tamoxifen.

In addition, groups 5 and 6 were similar to groups 3 and 4, but animals received  $0.5 \times 10^{11}$  of the Av3TATA-mEndo vector and  $1.5 \times 10^{11}$  of the C7LBD regulator vector, for a 1:3 ratio of target to effector. Groups 3 – 6 each contained no drug, 50  $\mu$ g, and 500  $\mu$ g tamoxifen treatment subgroups.

The results showed a dramatic induction of murine endostatin following the day 2 administration of 50  $\mu$ g of tamoxifen. The highest level of induction was observed on day 3, the day immediately following drug administration. Compared to the basal level observed on day 3 in the no tamoxifen groups, the C7LBDA(G521R) and C7LBDBs(G400V) regulators gave comparable fold induction, approximately 17 fold, and comparable absolute levels of expression, around 1500 ng/ml. In this study, the endogenous murine endostatin levels in an untreated mouse cohort was  $20 \pm 7$  ng/ml. The drug-induced endostatin expression rapidly declines by day 5, three days after drug administration, which is presumably due to the clearance of the tamoxifen and biological half life of the endostatin protein. In contrast, expression in the Av3RSV-mEndo treatment group persists at 200 ng/ml through day 15. In the 1:3 target to effector ratio groups, tamoxifen-induced expression reached 600 – 900 ng/ml, approximately 1/2 the level in the 1:1 ratio cohorts. This result indicates that *in vivo*, the transgene-containing vector, not the fusion protein-encoding vector, is limiting for absolute protein expression. Furthermore, endostatin expression in the animals treated with 500  $\mu$ g tamoxifen was comparable to the animals treated with only 50  $\mu$ g, indicating that the lower dose of tamoxifen is sufficient to fully activate the As(G521R) and Bs(G400V) regulators. Finally, the comparable low basal level of endostatin observed in the As(G521R) and Bs(G400V) groups suggests that the endogenous level of estrogen in the C57Bl/6

mice is not sufficient to induce the estrogen-responsive Bs(G400V) regulator. An elevation in basal endostatin levels observed at days 3 – 5 appeared to be a non-specific effect resulting from adenovirus vector administration, since the Av3Null vector has an effect similar to the Av3TATA-mEndo containing groups.

### Conclusions

The *in vitro* and *in vivo* results shown in this Example, demonstrate that the ZFP-LBD fusion proteins can be efficiently delivered via an adenovirus vector and can be expressed in sufficient amounts to provide high levels of drug-dependent control of a transgene in animals. Furthermore, the data show that the basal level of expression from the 6x2C7-minimal promoter constructs tested in an adenovirus vector give relatively low levels of expression, even when the fusion protein is expressed in the same cell. Thus, the system is highly drug dependent and allows for substantial regulation of the vector-delivered transgene. Taken together, these data evidence the effectiveness of this system for gene therapy applications.

### EXAMPLE 20

#### Construction and evaluation of the Cys<sub>2</sub>-His<sub>2</sub> Zinc finger DBD-ERLBD regulators in Lentiviral Vectors

In order to demonstrate controlled gene expression in an integrated vector system, the the regulatory system described in Example 19 with the adenoviral vectors were used to develop a series of lentiviral vectors. These vectors contained either the ZFP-LBD fusion protein linked to the immediate early CMV promoter or a regulatable transgene (either eGFP or luciferase) linked to the 6 X 2C7 array of C7 binding sites and either the minimal promoter from SV40 or C-fos TATA. The fusion protein-encoding vector and the regulatable transgene vector can then be used to generate lentiviral vector supernatant. The supernatant can be used to stably transduced human cells either singly or in parallel. Stable cell lines containing the integrated vectors can then be induced with the

appropriate activating drug (*e.g.*, 4-OH-tamoxifen) and gene expression is measured as fold induction in the presence and absence of drug.

**Construction of Lentiviral Vectors encoding the ZFP-LBD fusion protein or the regulatable transgene.**

The generation of lentiviral vectors and vector supernatant involves 3 main steps: first a gene or region of interest is inserted into shuttle vector backbone plasmid containing all of the viral cis-elements for transcription, packaging, reverse transcription, and integration. Second, the lentiviral vector shuttle plasmid is co-transfected into human 293 cells along with plasmids providing the packaging functions (gag, pol, and env). Typically the transfections include 10  $\mu$ g of vector plasmid, 10  $\mu$ g of packaging plasmid and 1  $\mu$ g envelope plasmid (Vesicular Stomatitis virus G envelope) using a Profection Calcium Phosphate transfection kit. Third, the culture supernatant containing the lentiviral vector is harvested (between 24 and 48 hours post transfection) and used to transduce naïve human target cells.

**Construction of HIV-1 based vectors**

An HIV-1-based vector system containing an internal CMV promoter was constructed from an infectious HIV-1<sub>IIIB</sub> provirus cDNA (pHIV-IIIB). The infectious proviral cDNA was generated by PCR from DNA isolated from H-9 cells chronically infected with HIV-1<sub>IIIB</sub>. The gag/pol and env sequences of pHIVIIIB were removed by digestion and excision of a PstI-KpnI fragment. Replacing the gag/pol and env sequences was a PstI/Kpn polylinker containing unique multiple cloning sites to form the intermediate vector p2XLTR. The Rev response element (RRE) fragment from HIVIIIB, required for proper vector RNA processing, was inserted downstream of the truncated gag sequences of p2XTR to form the construct pHIVec. An Asel-XbaI CMV-eGFP reporter fragment derived from pEGFP-N1 (Clontech, Palo Alto, CA) was cloned into the NdeI-Xba site of pHIVec to generate pHIVCMVGFP. pHIVCMV-X was

generated by removal of the eGFP fragment by KpnI digestion and religation.

#### **Construction of pHIVCMV-C7LBD/A(G521R)**

The AS521R (C7LBD/A(G521R)) coding fragment derived from C7LBDAS by digestion with NotI, T4 DNA polymerase fill-in, and EcoRI site was cloned into pHIVCMV-X cloned downstream of the CMV promoter into a EcoRI/SmaI restriction site. As a control for induction, an HIV vector containing a constitutive transactivator and DBD chimera was generated, pHIVCMV-C7VP16. A HindIII-NotI restriction fragment from pCDNA3-C7VP16 containing the C7VP16 coding fragment was inserted downstream of the CMV promoter at the Sma site of pHIVecCMV-X.

#### **Construction of pHIV6X2C7Sv and pHIV6X2C7TATA luciferase vectors**

A BamHI-XbaI restriction fragment containing the 6X2C7TATA luciferase fragment was isolated from pTATA6X2C7Luc and cloned downstream of the RRE at the SpeI-XbaI restriction sites. A MluI-BstBI restriction fragment containing the 6X2C7Sv luciferase fragment was isolated from pGL3-6X2C7SvLuc and cloned downstream of the RRE at the SpeI-XbaI restriction sites.

#### **Evaluation of the ZFP-LBD fusion proteins and regulatable lentiviral vectors**

##### **Transduction of HeLa cells by inducible lentiviral vectors**

Subconfluent HeLa cells were transduced with either HIV6X2C7SvLuc or HIV6X2C7TATALuc vector supernatant for 24 hours followed by transduction with HIVAS521R lentiviral vector supernatant. Cells were allowed to recover from infection for 24 hours in fresh culture medium after which 4-OH-tamoxifen (100 or 1000 nM) was added to the culture for an additional 24 hours. Cells were lysed in a standard luciferase lysis buffer, subjected to freeze thaw and analyzed for luciferase activity using a luciferase assay kit (Promega). The results showed that cells infected with either HIV6X2C7SvLuc or HIV6X2C7TATALuc followed by transduction with HIVCMVAS521R

resulted in a 13.1 and 11.7 fold stimulation in luciferase activity respectively, when given 4-OH-tamoxifen.

**Lentiviral Transduction of lentiviral integrated target vector populations**

HeLa cells that had been previously transduced with either HIV6X2C7SvLuc or HIV6X2C7TATALuc were carried in culture for 9 passages without exposure to any ZFP-LBD fusion protein. On passage 10, cells were transduced with HIVCMVAS521R for 24 hours followed by the addition of 100 nm tamoxifen for an additional 24 hours. The results show that HeLa cell lines containing an integrated HIV6X2C7SvLuc or HIV6X2C7TATALuc vector can be induced for luciferase expression by transduction of a LV containing AS521R+tamoxifen 31.4- and 22.5-fold, respectively.

These data demonstrate the effectiveness of the C2H2-LBD regulator for controlling expression of a transgene that is stably integrated into the host cell chromosome.

Since modifications will be apparent to those of skill in this art, it is intended that this invention be limited only by the scope of the appended claims.

**WHAT IS CLAIMED IS:**

1. A fusion protein, comprising a nucleotide binding domain operatively linked to a ligand binding domain derived from an intracellular receptor, wherein:

the nucleotide binding domain is a polydactyl zinc-finger peptide or modular portion thereof that specifically interacts with a contiguous nucleotide sequence of at least about 3 nucleotides; and

the fusion protein is a ligand activated transcriptional regulator.

2. The fusion protein of claim 1, further comprising an operatively linked transcription regulating domain.

3. The fusion protein of claim 1, wherein the intracellular receptor is a nuclear hormone receptor.

4. The fusion protein of claim 3, wherein the ligand binding domain derived from a nuclear hormone receptor has been modified to change its ligand selectivity compared to the native hormone receptor.

5. The fusion protein of claim 4, wherein the modified ligand-binding domain is not substantially activated by endogenous ligands.

6. The fusion protein of claim 1, wherein zinc-finger peptide binds to a sequence of nucleotides of the formula  $(GNN)_n$ , where G is guanine, N is any nucleotide and n is an integer from 1 to 6.

7. The fusion protein of claim 6, wherein n is 3 to 6.

8. The fusion protein of claim 1, wherein the zinc-finger peptide is comprised of modular units from a C2H2 zinc-finger peptide or a variant thereof that specifically interacts with a sequence of nucleotides and targets the fusion protein to a exogenous or endogenous gene that comprises the sequence of nucleotides.

9. The fusion protein of claim 1, wherein the zinc finger peptide is comprised of at least one zinc finger or a variant thereof that specifically binds to a targeted nucleic acid molecule.

10. The fusion protein of claim 9, that comprises at least three zinc fingers or variants thereof.

11. The fusion protein of claim 1, wherein the intracellular receptor is a nuclear hormone receptor selected from the group consisting of estrogen receptors, progesterone receptors, glucocorticoid- $\alpha$  receptors, glucocorticoid- $\beta$  receptors, mineralocorticoid receptors, androgen  
5 receptors, thyroid hormone receptors, retinoic acid receptors, retinoid X receptors, Vitamin D receptors, COUP-TF receptors, ecdysone receptors, Nurr-1 receptors and orphan receptors.

12. The fusion protein of claim 1, wherein the intracellular receptor is a steroid receptor.

13. The fusion protein of claim 4, wherein the hormone receptor is a progesterone receptor variant or an estrogen receptor variant, wherein a receptor variant comprises a ligand binding domain that has selectivity and sensitivity for endogenous and exogenous ligands that differ from its native ligands.

14. The fusion protein of claim 2, wherein the transcription regulating domain comprises a transcription activation domain.

15. The fusion protein of claim 2, wherein the transcription regulating domain comprises a transcription activation domain selected from the group consisting of VP16, VP64, TA2, STAT-6, p65 and derivatives, multimers and combinations thereof that have transcription activation activity.

16. The fusion protein of claim 14, wherein the transcription regulating domain comprises a nuclear hormone receptor transcription activation domain or variant thereof that has transcription activation activity.

17. The fusion protein of claim 14, wherein the transcription regulating domain comprises a steroid hormone receptor transcription activation domain or variant thereof.

18. The fusion protein of claim 14, wherein the transcription regulating domain comprises a viral transcription activation domain or variant thereof that has transcription activation activity

19. The fusion protein of claim 18, wherein the transcription regulating domain comprises a VP16 transcription activation domain or variant thereof.

20. The fusion protein of claim 2, wherein the transcription regulating domain comprises a transcription repression domain.

21. The fusion protein of claim 20, wherein the transcription repression domain is selected from the group consisting of ERD, KRAB, SID, Deacetylase, and derivatives, multimers and combinations thereof such as KRAB-ERD, SID-ERD, (KRAB)<sub>2</sub>, (KRAB)<sub>3</sub>, KRAB-A, (KRAB-A)<sub>2</sub>, (SID)<sub>2</sub> (KRAB-A)-SID and SID-(KRAB-A).

22. The fusion protein of claim 2 encoded by the sequence of nucleotides set forth in any of SEQ ID Nos. 1-18.

23. A nucleic acid molecule, comprising a sequence of nucleotides encoding the fusion protein of claim 1.

24. A nucleic acid molecule, comprising a sequence of nucleotides encoding the fusion protein of claim 2.

25. The nucleic acid molecule of claim 23, wherein the the fusion protein is encoded by a sequence of nucleotides set forth in any of SEQ ID Nos. 1-18.

26. A vector, comprising a sequence of nucleotides encoding the fusion protein of claim 1.

27. A vector, comprising a sequence of nucleotides encoding the fusion protein of claim 2.

28. A cell, comprising the expression vector of claim 26.

29. A cell, comprising the expression vector of claim 27.

30. The cell of claim 28 that is a eukaryotic cell.

31. The cell of claim 29 that is a eukaryotic cell.

32. The vector of claim 26 that is a viral vector.

33. The vector of claim 27 that is a viral vector.

34. The vector of claim 32, wherein the viral vector derived from a DNA virus or a retrovirus.

35. The vector of claim 34 that is selected from the group consisting of an adenoviral vector, and adeno-associated viral vector, a herpes virus vector, a vaccinia virus vector and a lentiviral vector.

36. The vector of claim 33 that is a viral vector.

37. The vector of claim 36, wherein the viral vector derived from a DNA virus or a retrovirus.

38. The vector of claim 37 that is selected from the group consisting of an adenoviral vector, and adeno-associated viral vector, a herpes virus vector, a vaccinia virus vector and a lentiviral vector.

39. A combination, comprising:  
a fusion protein of claim 1 or a nucleic acid molecule comprising a sequence of nucleotides that encodes the fusion protein;  
and

a regulatable expression cassette that comprises at least one response element recognized by the nucleic acid binding domain of the fusion protein.

40. The combination of claim 39, wherein the cassette comprises a gene that encodes a therapeutic product.

41. The combination of claim 39 that comprises a single composition that contains the fusion protein or nucleic acid molecule that encodes the fusion protein, and the regulatable expression cassette in a pharmaceutically acceptable excipient.

42. The combination of claim 39, wherein the fusion protein or nucleic acid molecule comprising a sequence of nucleotides that encodes the fusion protein, and the regulatable expression cassette are in separate compositions.

43. A composition for regulating gene expression comprising:  
an effective amount of the fusion protein of claim 1 or a nucleic acid molecule comprising a sequence of nucleotides that encodes the fusion protein; and  
a pharmaceutically acceptable excipient.

44. The composition of claim of claim 43 that is formulated for single dosage administration.

45. A composition for regulating gene expression comprising: an effective amount of the fusion protein of claim 2; and a pharmaceutically acceptable excipient.

46. The combination of claim 39, wherein the regulatable expression cassette comprises 3 to 6 response elements.

47. A method for regulating gene expression in a cell, comprising:

introducing into a cell a fusion protein of claim 1 or a nucleic acid molecule that comprises a sequence of nucleotides that encodes the fusion protein; and

contacting the cell with a ligand that interacts with the binding domain in the fusion protein, whereby the fusion protein interacts with a target nucleic acid molecule to activate or repress transcription of a gene encoded by the fusion protein.

48. The method of claim 47, wherein the ligand binding domain is modified whereby it interacts with a non-natural ligand.

49. The method of claim 47, wherein the target nucleic acid molecule is endogenous to the cell.

50. The method of claim 47, wherein the target nucleic acid molecule is introduced to the cell as part of an expression cassette.

51. The method of claim 47, wherein the expression cassette and fusion protein or nucleic acid encoding the fusion protein are introduced at the same time.

52. The method of claim 47, wherein the expression cassette and fusion protein or nucleic acid encoding the fusion protein are introduced sequentially.

53. The method of claim 47, wherein ligand is delivered to the cell after the fusion protein or nucleic acid molecule encoding the fusion protein is introduced into the cell.

54. The method of claim 47, wherein the nucleic acid molecule encoding the fusion protein comprises a vector.

55. The method of claim 54, wherein the vector is a viral vector.

56. The method of claim 47, wherein the cell is in a mammal.

57. The method of claim 50, wherein the expression cassette is contained in a vector.

58. The method of claim 57, wherein the vector is a viral vector.

59. The method of claim 50, wherein the cell is in a mammal.

60. The method of claim 47, wherein the ligand binding domain derived from a nuclear hormone receptor has been modified to change its ligand selectivity compared to the native hormone receptor.

61. The method of claim 60, wherein the modified ligand-binding domain is not substantially activated by endogenous ligands.

62. The method of claim 47, wherein zinc-finger peptide binds to a sequence of nucleotides of the formula  $(GNN)_n$ , where G is guanidine, N is any nucleotide and n is an integer from 1 to 6.

63. The method of claim 62, wherein n is 3 to 6.

64. The method of claim 47, wherein the zinc-finger peptide is comprised of modular units from a C2H2 zinc-finger peptide or a variant thereof that specifically interacts with a sequence of nucleotides and targets the fusion protein to a exogenous or endogenous gene that comprises the sequence of nucleotides.

65. The method of claim 47, wherein the zinc finger peptide is comprised of at least one zinc finger or a variant thereof that specifically binds to a targeted nucleic acid molecule.

66. The method of claim 65, that comprises at least three zinc fingers or variants thereof.

67. The method of claim 47, wherein the intracellular receptor is a nuclear hormone receptor selected from the group consisting of estrogen receptors, progesterone receptors, glucocorticoid- $\alpha$  receptors, glucocorticoid- $\beta$  receptors, mineralocorticoid receptors, androgen

receptors, thyroid hormone receptors, retinoic acid receptors, retinoid X receptors, Vitamin D receptors, COUP-TF receptors, ecdysone receptors, Nurr-1 receptors and orphan receptors.

68. The method of claim 47, wherein the intracellular receptor is a steroid receptor.

69. The fusion protein of claim 1, wherein the polydactyl zinc-finger peptide or modular portion thereof specifically interacts with a contiguous nucleotide sequence of at least about 3 nucleotides to about 18 nucleotides.

70. A non-viral delivery system, comprising the fusion protein of claim 1 or a nucleic acid molecule encoding the fusion protein.

71. The non-viral delivery system of claim 70, further comprising a nucleic acid molecule that comprises an expression cassette containing a sequence of nucleotides with which the nucleic acid binding domain of the fusion protein interacts.

72. The non-viral delivery system of claim 70, wherein the non-viral delivery system is selected from the group consisting of DNA-ligand complexes, adenovirus-ligand-DNA complexes, direct injection of DNA,  $\text{CaPO}_4$  precipitation, gene gun techniques, electroporation, liposomes and lipofection.

73. The fusion protein of claim 9, wherein the zinc finger peptide comprised of at least one zinc finger or a variant thereof specifically binds to a targeted nucleic acid molecule with a dissociation constant of less than about 1.0 nanomolar.

**ABSTRACT**

Fusion proteins for use as ligand-dependent transcriptional are provided. The fusion proteins include a nucleotide binding domain operatively linked to a ligand-binding domain. They also can include a transcription regulating domain. The nucleotide binding domain is a zinc-finger peptide that binds to a targeted contiguous nucleotide sequence of from 3 to about 18 nucleotides are provided. The fusion proteins are used for gene therapy. Also provided are polynucleotides encoding the fusion proteins, expression vectors, and transfected cells.

5

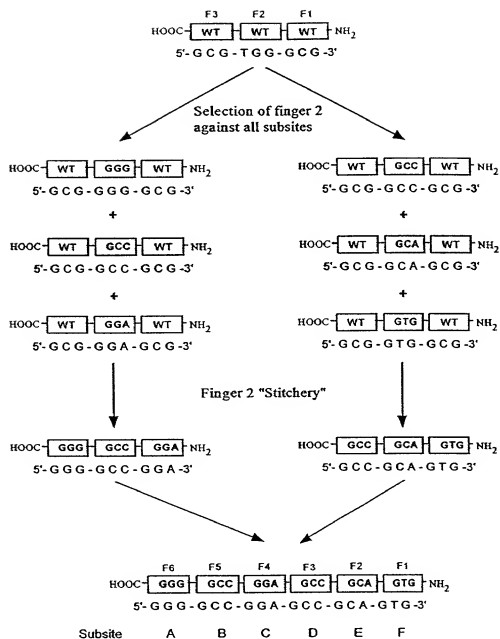


FIGURE 1

# Domains of a Steroid Nuclear Receptor ex. Human Estrogen Receptor (ER)

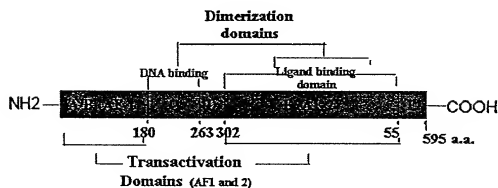


FIGURE 2

# Cloning Strategy for DNA binding domain Replacement

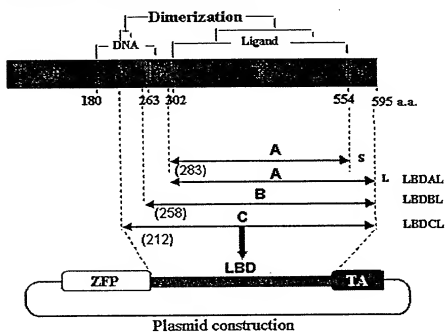


FIGURE 3

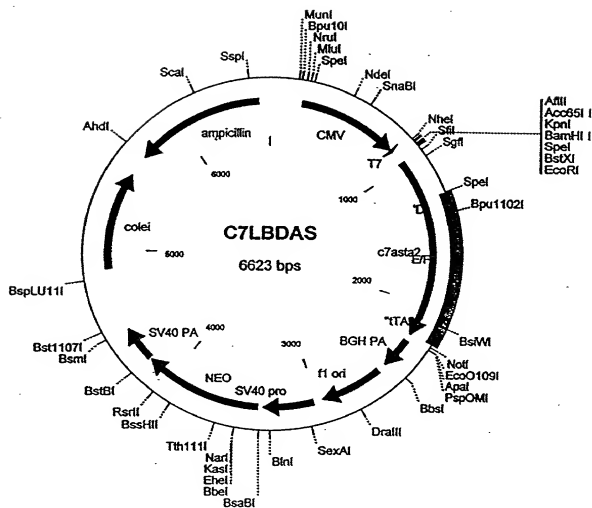


FIGURE 4





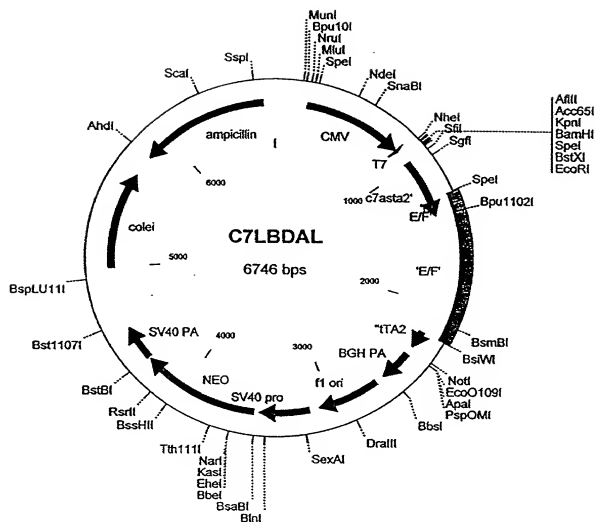


FIGURE 7

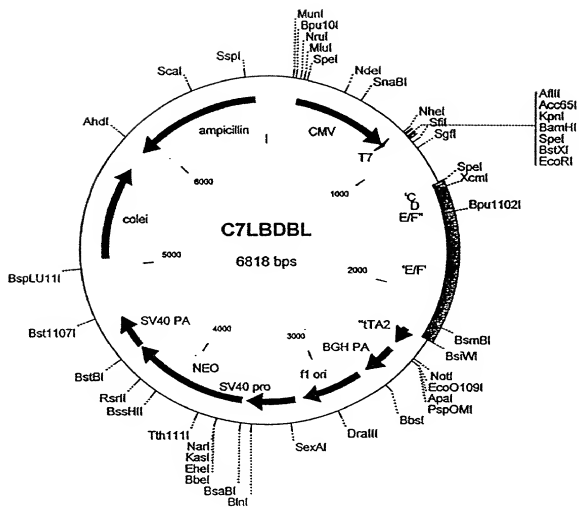


FIGURE 8

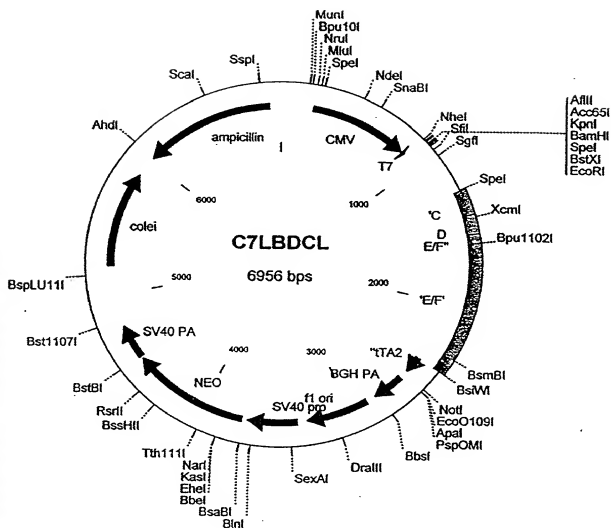
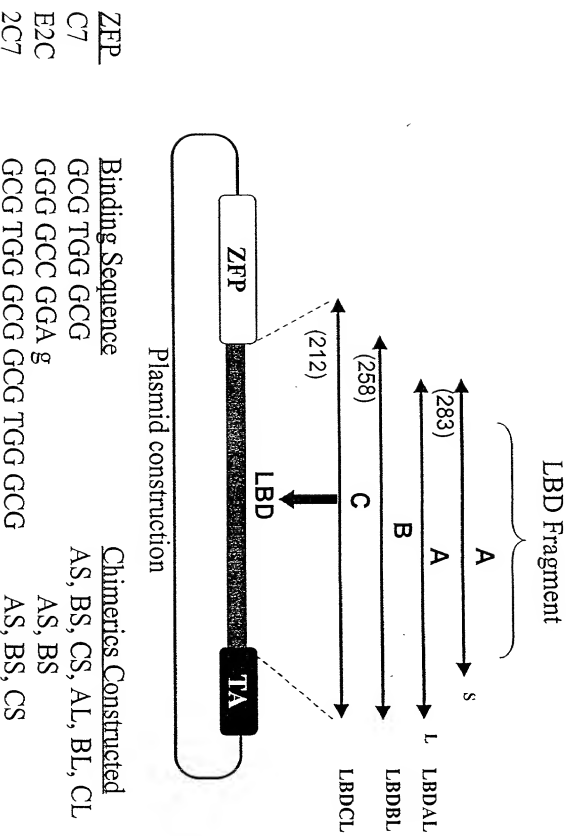


FIGURE 9



**FIGURE 10**



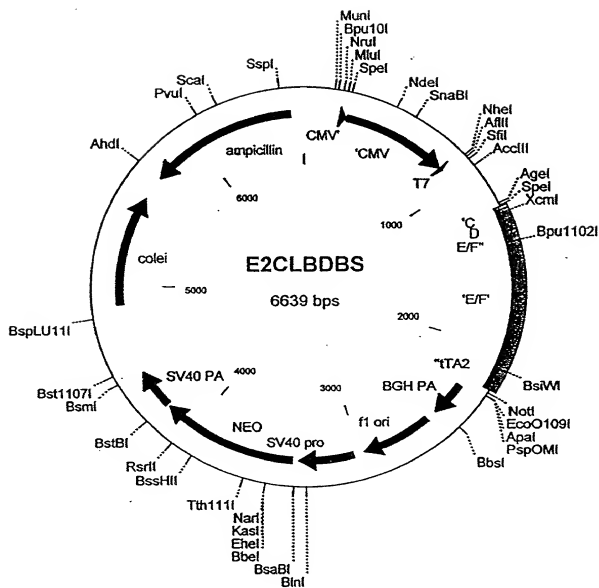
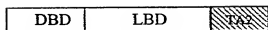
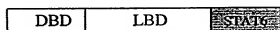


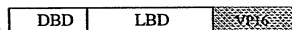
FIGURE 12



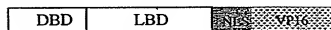
C7ASTA2 or C7BSTA2



C7BS-



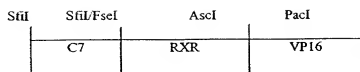
C7BSVP16



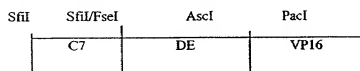
C7ASNLVP16

FIGURE 13

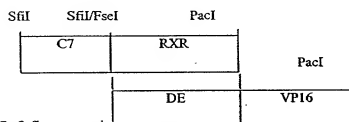
002207-52392920



C7-R-VP16



C7-DE-VP16



C7-R

DE-VP16

C7: 3-finger protein  
 RXR: mammalian RXR ligand binding domain  
 DE: EcR ligand binding domain from *Drosophila*  
 BE: EcR ligand binding domain from *Bombyx mori*

FIGURE 14

002020.5233555

### Subcloning for 2C7-LBD expression constructs

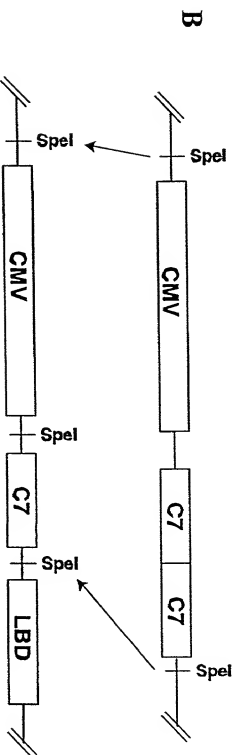
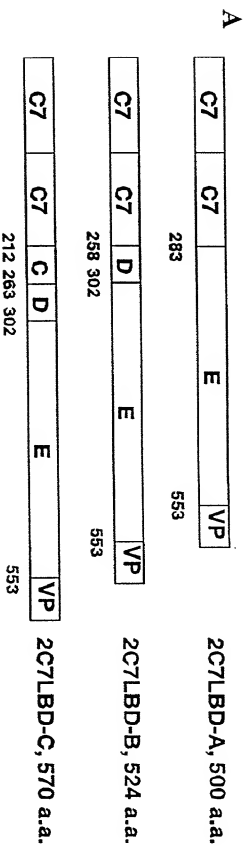


FIGURE 15

03456789 0123456789

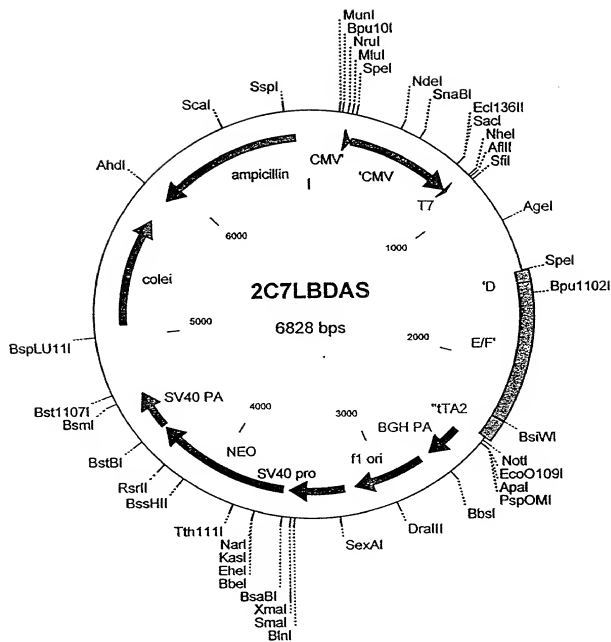


FIGURE 16

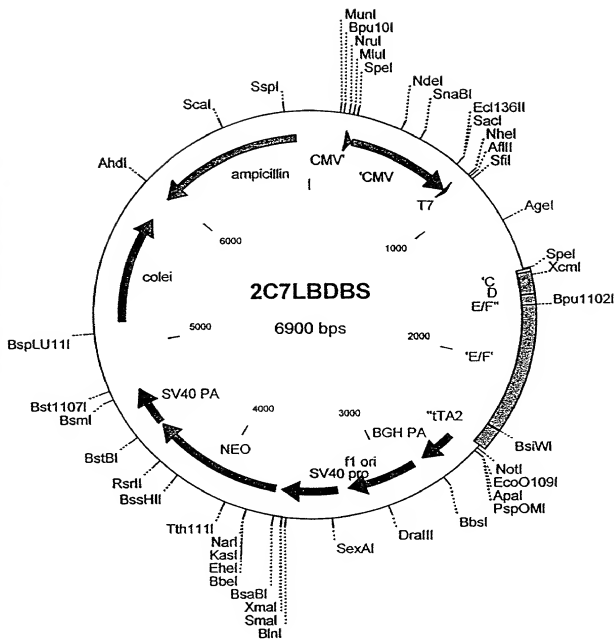


FIGURE 17

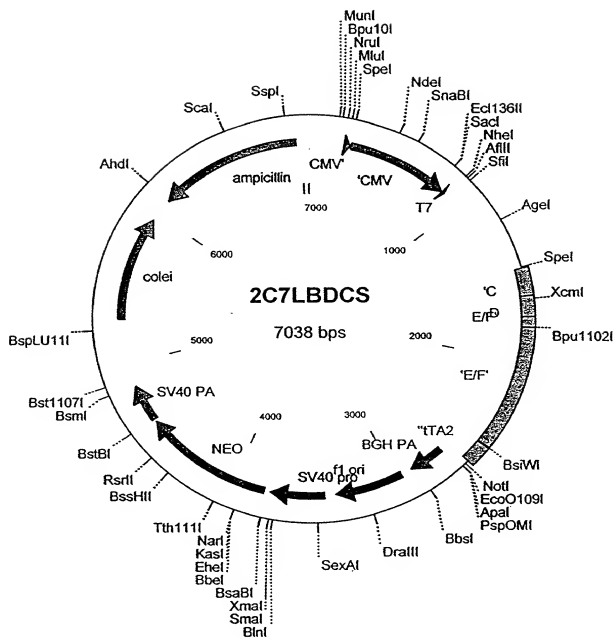


FIGURE 18

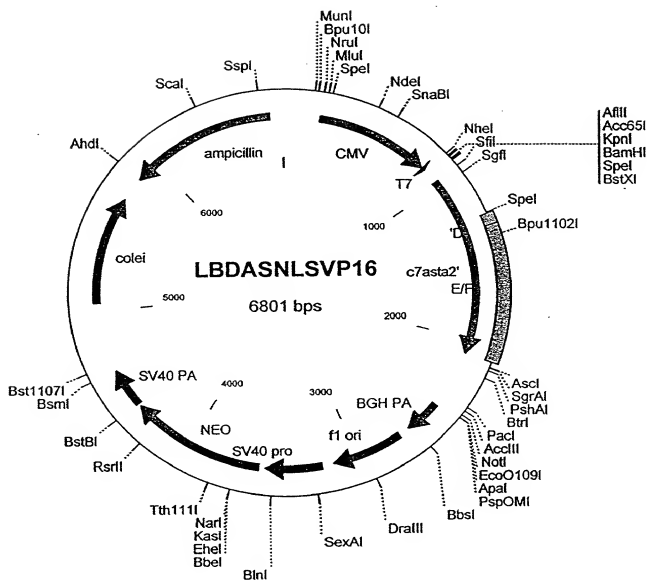


FIGURE 19

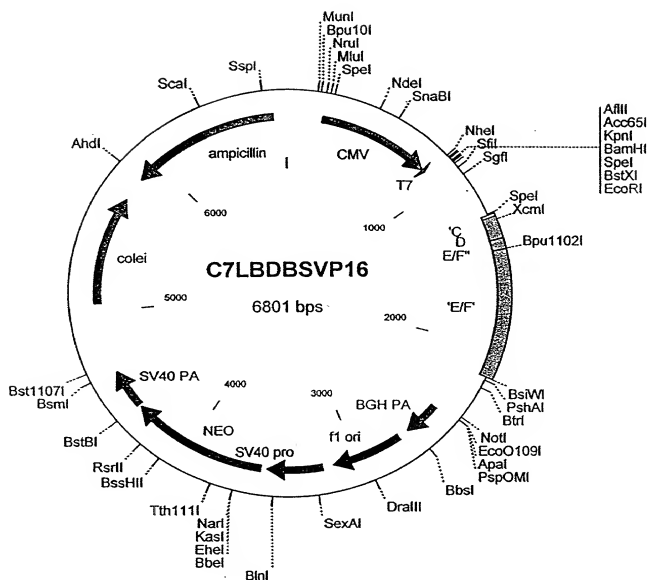


FIGURE 20

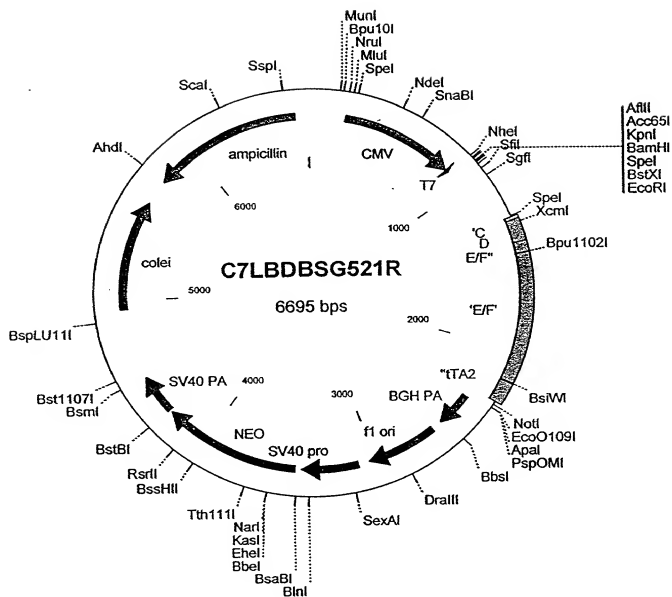


FIGURE 21

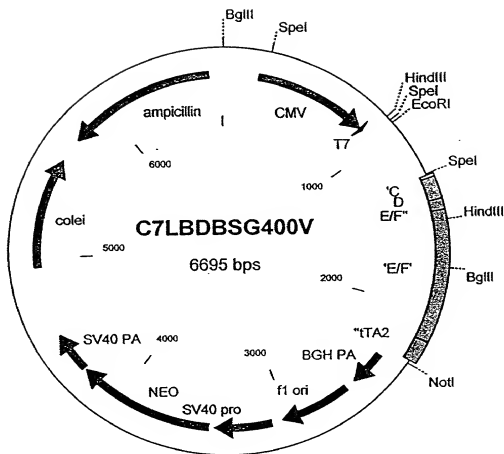
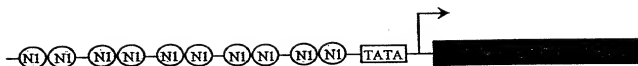


FIGURE 22

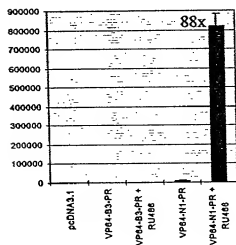
A



**(N1-N1)** = GGG GTA GAA AAA GGG GTA GAA

B

RU486



C

Tamoxifen

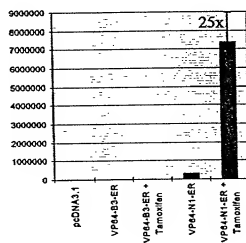


FIGURE 23

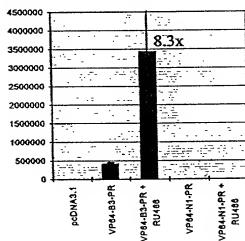
A



●● = GGA GGG GAC AAA GGA GGG GAC

B

RU486



C

Tamoxifen

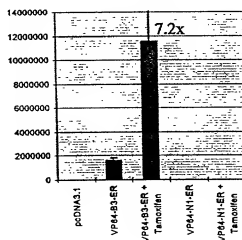
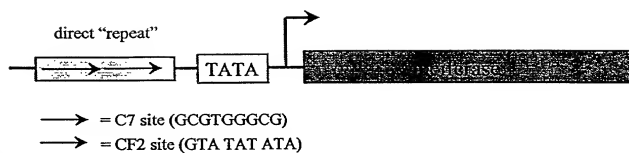


FIGURE 24

A



B

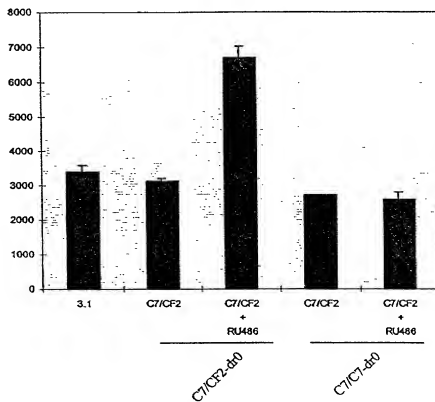


FIGURE 25

# Left end shuttle plasmid for adenovirus vector construction

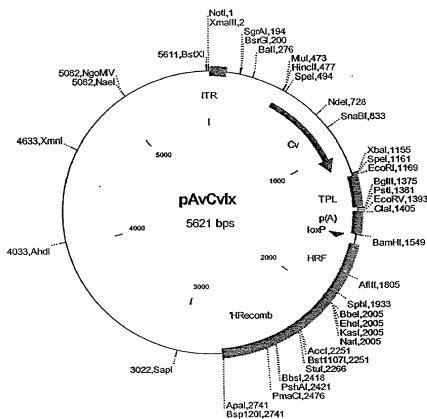


FIGURE 26

Figure 27, Right End Vector Genome-containing plasmid

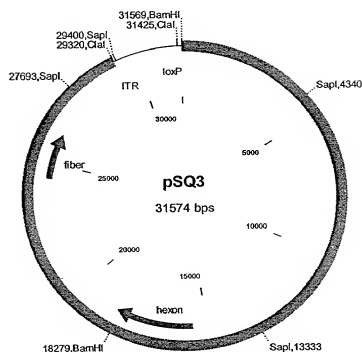


FIGURE 27

002030-52938560

# SEQUENCE LISTING

<110> Barbas, Carlos F., III  
Kadan, Michael  
Beerli, Roger

<120> LIGAND ACTIVATED TRANSCRIPTIONAL REGULATOR PROTEINS

<130> 22908-1227B

<140> Unknown

<141> 2000-06-02

<150> 09/433,042

<151> 1999-10-25

<160> 92

<170> PatentIn Ver. 2.0

<210> 1

<211> 6828

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Construct  
2C7LBDAS

<400> 1

```

gacggatcgg gagatctccc gatcccccatt ggtcgactct cagtacaatc tgcctctgatg 60
cgccatagtt aagccagttat ctgctccctg cttgtgtgtt ggaggtcgct gactagtcgc 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc 180
ttagggttag cgcgttttgc ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaataaa ttacggggct attagttcat agccatata 300
tggagtctcg cgttacataa cttaacgtta atggcccgcc ttgctgaccg cccaacgacc 360
cccgccattt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc 420
attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt 480
atcatatgccc aagtacgccc cctattgacg tcaatgacgg taaattggccc gctggcatt 540
atgcccgata catgacctta tgggaacttc ctacttgcca gtacatctac gtattagtc 600
tcgctattac catggtgatg cgggttttgc agtatacaaa tgggcgttga tagcgggttg 660
actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc 720
aaaatcaacg ggactttcca aaatgtcgta acaactccgc cccattgacg caaatggggc 780
gtaggcgtgt acggtgggag gtctatataa gcagagctct ctggcttaact agagaccaca 840
ctgcttactg gcttatcgaa attaatcaga ctactatag ggagaccaca gctgctatgc 900
gtttaaactt aagcttagat ctatggccca ggcggccctc gagccctatg tctgccctgt 960
cgagtcctgc gatcgccgct tttctaagtc ggcgtgatct aagcgccata tcgcgatcca 1020
cacaggccag aagcctttcc cccacacagg gaggcatacc aacttcagtc gtagtgacca 1080
ccttaccacc cccaggagtg atgaacgcaa cgcgcgctt tctaatgctg acattgtgtg 1140
gaggaagttt gcccctgtcg agtcctcgca caggccagaa gcccttcag ttctcgatga 1200
gcgcataatc agtcacacca ttaccacca catccgcacc caacaggcg agagccttt 1380
cttcagctgt attttggga ggaagtttgc caggagtgtg gaacgcaaga ggcataccaa 1440
tgccctattt aagacagagg actctagaac cagtctctgt ggagacatga gagctgcaca 1500
cctttggcca agcccgctca tgatcaaacg ctctaaagaag aagcctctgg cctgtccct 1560
gaacggcgag cagatgtca gtgcctgtgt ggaatgctga cccccaac tctattcga 1620
gtatgatcct accagacccct tcatgaagc ttctgatgat ggcctactga ccaactggc 1680
agacaggagg ctggttcaaa tgatcaactg ggccgaagag gtgcacagct tttggagatt 1740
gacctctcat gatcaggtcc acctctatga atgtgctggt ctatagatct tgaatgttg 1800
ctctgtctgg cgctccatg agcaccgag gaagctactt ttgtctccta acctgctct 1860
ggacaggaaac cagggaaaat gtgtagagg catggtggag atcttcgaca tgcctgtggc 1920

```



ccatctggcc	ccagtgctgc	aatgataccg	cgagaccacg	gctcaccggc	tccagattta	6000
tcagccataa	accagccacg	cggaaagggc	gagcgcagaa	gtgggtctgc	aactttatcc	6060
gcctccatcc	agctctattaa	ttgtttgcgg	gaagctagag	taagttagtc	gccagttaat	6120
agttttgcga	acgtttgttc	cattgtctaca	ggcatcgtgg	tgctcacgctc	gcgcttggg	6180
atggcttcat	tcagctccgg	ttcccaacga	ccaaggcgag	ttacatgtctc	cccctgtgtg	6240
tgcataaaag	cggtttgatgc	cttcggtcct	cagatcgttg	tcagaagtaa	gttggccgca	6300
gtgtttatcac	tcattggttat	ggcagcactg	cataattctc	ttactgttcat	gccatccgta	6360
agatgctgtt	ctgtgactgg	tgagtactca	accaagtcat	tctgagaata	gttatggcgg	6420
gcagcaggtt	gctctttgcc	ggcgtcaata	cgggataata	ccgcgcacaa	tagcagacct	6480
ttaaaaagtc	tcattcatgg	aaaaacgttct	tcggggcgaa	aactctcaag	gatcttaccg	6540
ctgttgagat	ccagttcgat	gtaacccactc	cgtgcaccca	actgatcttc	agcatctctt	6600
actttcacca	gctttcttcg	gtgagcaaaa	acaggaagcg	aaaaatccgc	aaaaaaggga	6660
ataagggcga	ccaggaaatg	ttggaatact	atactctctc	tttttcaata	ttatgtgaag	6720
attatccagg	gtttattgtct	catgagcgga	tacatatctt	aatgtattta	gaaaaataaa	6780
caaatagagg	ttccgcgcac	atttccccga	aaagtgcac	ctgacgttc		6828

<210> 2  
 <211> 6900  
 <212> DNA  
 <213> Artificial Sequence

<220>

<220>  
 <223> Description of Artificial Sequence: Construct  
 2C7LBDBS

<400> 2	gatccccctat	ggtcgactct	cagtacaatc	tgctctgatg	60
gacggatcgg	ctgctccctg	cttgtgtgtt	ggaggtgcgt	gagtagtcgc	120
ccgcatagtt	acaaggcga	gcttgaccga	caattgcatg	aagaatctgc	180
tcagcaaaa	ctgcttccgc	ctgttacggc	cagatatacg	cggttagact	240
ttagggttag	tagtaatacaa	ttacgggttc	attagttcat	agcccatata	300
gattattgac	cttacggttaa	atggcccgcc	tggtgtgacg	cccaaacgac	360
gtgagttcgc	atgacgtatg	ttcccatagt	aacgcacaat	gggaactttc	420
cccgcccat	tattttacggt	aaactgcaca	cttggcagta	catcaagtgt	480
atgacgtcca	cttatgtgacg	ctaatgacgg	taaatggccc	gcttggcatt	540
atcatatgac	tgggactttc	ctacttgcca	gtacatctac	gtattagcca	600
atgcccgatg	cggttttggc	agtagctcaa	tgggcggtga	tagcggtttg	660
tcgctattac	ctccacccca	acaactccgc	tgaggagtgt	ttttggcacc	720
actcacgggg	aaatgtcgta	gcagagctct	cccattgacg	caaatggggc	780
aaaatcaag	gtctatataa	ctcactatag	gagccctatg	agagaacca	840
gtaggcggtg	attataacga	ggcgccctc	ggagacccaa	gctggctagc	900
ctgtcttactg	ctatggccca	ggctgatctg	aaacttcagt	tgatgtacca	960
ttttaaactt	tttctaagtc	atgctgcagt	tttgctctgt	acatttgtgt	1020
cgagtccctg	aggtgtcgaat	cgagaagctc	aacttcagtc	gcgtagtcca	1080
cacaggcgag	ccccacacgg	atgaaatcac	tctaatgcgg	ctgatgtgaa	1140
ctctaccacc	atgaaacgaa	tcgcgcgttt	tgctcgaat	catagtggtaa	1200
gaggaagtgt	agctctcgca	gcccctccag	catccgcacc	caacacaggg	1260
gcccataatc	ttaccaccca	cagcgcagaa	caggagtgat	ggacgcgaag	1320
ctctcagtgt	ggaagtgttc	tagtgaccga	atgaggggga	gaatgttgaa	1380
tgccctgtgac	actctagaac	cagggttgaa	gtgggtgtcg	ctggagacat	1440
ataccattta	atggggaggg	catgatcaaa	tttggtgtcg	agaaacagct	1500
acacaagcgc	caagcccgct	cagtgctctg	cttcagtgaa	tgggcggaag	1560
gagagctgcc	accagatggt	cttcagtgaa	tgggcggaag	gggtggcagg	1620
ggccttgtcc	ctaccagacc	catgatcaaa	gggaagctac	agatttctgc	1680
actctatttc	agctgggttca	ccacctctta	ggatgtgctg	cgatgtcgag	1740
gaccacactg	atgatcaggt	ggagcaccga	ctgcaggagg	aggagtttgt	1800
cttttggtgat	ggcgctccat	cttcagtgaa	acatttctgt	ccagacacct	1860
ctctgatgatt	gtgctctgtc	catgatgaat	gagatctctg	cagacacttt	1920
taacttgctc	cttgacacga	tggaagtgtac	gacacagatca	ggctggccca	1980
catgctgtgat	gctacatcat	ccgagtcctg	cagcagccag	agcatctgta	2040
gtgcctcaaa	tgcttatttt	cctgcagcag	aaagcgatgg	tgtctggacg	2100
gaagtctctg	gaagagaagg	catgatgaac	ctgctggaga		2160
gactcaactg	caggcctgac	ctgctgacct			2220
gctcctctct	acatcaggga				2280
cagcatgaag	tggtgcccc				2340

ccaccgccta	catgcgccta	ctagccgtac	gccggccgac	gccctggagc	acctcgacct	2400
ggacatgctg	ccggcccgacg	ccctggagca	cttcgacctg	gacatgctgc	cgggccgacg	2460
ctcggagcag	ctcggactgg	acatgctgcg	ggggtaacta	agtaaacggc	cgctcgagtc	2520
tagtagggcc	gttttaaaccc	gctgatcagc	ctcgcactgt	cccttcgattg	cgacgacatc	2580
tgtgttttgc	ccctcccccgc	tgctctccct	gacccctggaa	ggtgccactc	ccactgtccct	2640
ttcctaataa	aatgaggaaac	ttgcatcgca	ttgtctgagt	agggtgcatt	ctattctcggg	2700
gggtgggggt	ggggcaggaca	gcaaggggga	ggatggggaa	gacaatgagc	ggatgctggc	2760
ggatggcggt	ggctctatagg	cttctgaagg	ggaaagaaac	agcctggggc	ctagggggta	2820
ttcccacggt	ccctgtatggc	gcgcataagg	cgcgggcggt	gtgggtggta	cgccgagcgt	2880
gacccgtata	ctgtcccgagc	ccctagcgcc	cgctcccttcc	gctttcttccc	cttcccttccc	2940
ggccacgttc	ctcgtctcttc	cccgctcaagc	ctctaactcgg	ggcatccctt	tagggttccg	3000
atttagtgct	ttacgggcacc	ttgcaccccaa	aaaacttgat	taggggtgatg	gttccactgat	3060
tggggccatc	ccctgatgaga	cggttttttcg	cccttttgagc	ttggagtcca	cgctctcttaa	3120
tagtgactgc	ttgtccaaaac	ctggaaacaac	actcaacctc	ctcgcgcttc	attctcttaa	3180
tttataaggg	atttttgggga	tttcggccta	ttgtgttaaaa	aatgagctga	tttaacaaa	3240
aatataacgc	aattattctc	gtggaattgt	ttgtcagttag	gtgttggaac	gtccccagcg	3300
ttcccaggca	ggcagaaatg	ttgcaaaagc	gcactctcaat	atgctctca	ccaggtgtgg	3360
aaagtcccca	ggctcccag	caggcagaag	tatgcaaaagc	atgatctcca	atttagtcag	3420
aaccatagct	ccgcgccctaa	ctccgcccat	cccgcccccta	actccgcctca	gttccgcctca	3480
ttctccggcc	catggctgac	taattttttt	tattttatga	gaggccgagc	cgccctctgc	3540
ctctgagcta	ttccagaagt	agtgaggagg	cttttttgga	ggcctaggct	tttgcaaaaa	3600
gtccccggct	gcttgatatt	ccattttcgg	atctgatcaa	gagacaggat	gaggtatgct	3660
tcgcattgatt	gaaocaagat	gattgcacgc	agggtctccgc	cgccgtctggg	ttgagagagct	3720
atttcggctat	gactggcgac	aacagacaat	cggtcgtctc	gatgcgccgc	tgttccgcgt	3780
gtccagccgag	ggggcccgccg	ttctttttgt	caagaccgac	ctgtccgggt	ccctgaatga	3840
actgcaggag	gaggccagcg	ggctatcgtg	gctggccacg	accggcgctc	cttgcgcagc	3900
tgtgtctcgac	gtttgtcactg	aagcggggaag	ggactggctg	ctatttgggc	aaagtccggg	3960
gcaggatctc	ctgtcatctc	acccttgcctc	tgccgagaaa	gtatccatca	tggtgatgac	4020
taacggcgcg	ctgcatacgc	ttgatccggc	tacctgccca	ttcgaccacc	aagccgaaca	4080
ccgctatcgag	cgagcacgta	ctcggtatgga	agccggtctt	ctgcatacag	atgatctgga	4140
ggaagagcat	caggggccctcg	cgccacgcga	actgttcgcc	aggctcaagg	cgccgatccct	4200
cgacggcgag	gatctcgtcg	tgacccatgg	cgatgcctgc	ttgcctaata	tcaagggtga	4260
aaatggccgc	ttttctggat	ttcatgactg	ttggccggctg	gggtgtggcg	accgcgtata	4320
ggacatcgct	ttggctaccc	tgtgatattg	tgaagagctt	ggcgccgaat	gggctgacgc	4380
cttctcctgt	ctttacggta	tcgcgcctcc	cgattcgcag	cgcatcgccct	ctcatcgctt	4440
ttcttgacgag	ttctcttgag	cgggactctg	gggtttcgaaa	tgaccgcgca	tgccagccgc	4500
aaacctgccat	cacgagattt	cgattccacc	gccgcctctc	atgaaaggtt	gggcttcgga	4560
atcgtttttc	gggacccggg	ctggatgatt	ctccagccgc	gggatctcat	gctggatgtc	4620
ttcgcccaac	ccaacttggt	tattgcagct	tataatgggt	acaaatgaag	caatagcatc	4680
acaaaattca	caaataaagc	atttttttca	ctgcattcta	gtgtgagctt	gtccaaaactc	4740
atcaatgtat	ctttatcatg	ctgtataacc	tgcactctca	gctagagctt	ggcgtaatca	4800
tggctcatag	gtgaaattgt	gtgaaattgt	tatccgctca	caattccaca	caacatacga	4860
gcgggaagca	taaaagtgtaa	aggctggggg	gcctaatgag	tgagctaaact	cacattaat	4920
gcgttgcgct	cactgcggcg	tttccagtgc	ggaaaactgt	cgctgccagct	gcattaatga	4980
atcgcccaac	gcggcgggag	aggcgggttg	cgccgagggc	tatcagctca	ctcaaaaggc	5040
gtataacggg	tatccacaga	atcaggggat	aacgcagaaa	agaaactatg	agcaaaaaggc	5100
cagcaaaaag	ccagggaaccg	taaaaaggcc	gcgttgcctg	cgttttttca	taggctccgc	5160
ccccctgagc	agcatcacaga	aaaatgcacg	agctccctgc	gttgggggaa	cccgagcaga	5220
ctataaagat	acragcggtt	ttcccctgga	ctcccttcgg	tgccgctctc	tgcttcggac	5280
ctcgccgtta	ccggtataact	gtccgccttt	taggtcgttc	gaagcgtggc	gctttctcaa	5340
tgctcacgct	tgatgatatt	cagtttcggg	gccttatccg	gtcccaagct	gggctgtgtg	5400
cacgaaaccc	ccgcttcagcc	cgaccgcctg	gcagcagcca	gtgtataacg	tcttgagctc	5460
aaocccggtaa	acagcagact	atcgccactc	ttgaagtgtg	ggcctaacta	cggtctacat	5520
gcgaggtatg	taggcggtgc	tacagagttc	ctgagccag	ttaccttcgg	aaaaagggtt	5580
agaaagcacg	tatttgcgtc	ctgcgctctg	gctggtagcg	gtgggttttt	tgtttgcag	5640
ggtagctctt	gctgcggcga	acaaaccacc	caagaagatc	ctttgatctt	tttccagggc	5700
cagcagcatt	cgccgcagaaa	aaaaggatct	taagggattt	ttggtcatgag	attatcaaaa	5760
cttgacgctc	agtggaacga	aaactcaact	aaatgaagt	ttaaattcaat	ctaaagtata	5820
aggatcttca	ctctggtctga	cagttaccaa	tgcttaatac	gtgagggcacc	tattctagcg	5880
tatgagtata	ttcgttcaat	catagttgcc	tgactcccg	tcgtgtagat	aactacagca	5940
atctgctctat	taccattctat	ccccagtgcc	gcaatgatac	ccgcgagacc	acgctcaccg	6000
cgggagggct	tatcagaagt	aaaccagcca	gccgggaagg	ccgagccgag	aagtggtccct	6060
gctccagatt	cgccctccat	ccagttctatt	aattgttggc	gggaagtgta	agtaagtatg	6120
cgcaactttat	atagtttgcg	caacggttgt	gccattgcta	caggcatcgt	ggtctcacgc	6180
tcggcagtta	gtatggcttc	attcagctcc	ggttcccaac	ctcgaaggcg	agttaacatg	6240
tcgtcgcttg	tgctcaaaa	agcgggttagc	tccttcggct	atgatcagct	tgctcagaag	6300
tgctcgaagt						6360

aagttggcgc	cagtggttacc	actcatgggt	atggcagcac	tgcaataatc	tcttactgtc	6420
atgccatcgc	taagatgctt	tctgtgact	ggtagactac	caaccaagtc	attctgagaa	6480
tcgtgtatgc	ggcgaccgag	ttgctcttgc	cggcgctcaa	tacgggataa	taccgcgcca	6540
catagcagaa	ctttaaaaagt	gctcatcatt	ggaaaaagct	cttcgggggc	aaaactctca	6600
aggatctttac	cgcgttttgag	atccagttcg	atgtaaacca	ctcgtgcacc	caactgatct	6660
tcagatcttt	ttaactttcac	cagcgtttct	gggtgagcaa	aaacaggagaa	gcaaaatgcc	6720
gcaaaaaagg	gaataagggc	gacacggaaa	tgttgaatac	tcatactctt	ctcttttcaa	6780
tattattgaa	gcatttatca	gggttattgt	ctcatgagcg	gatacatatt	tgaatgtatt	6840
tagaaaaata	aacaaatagg	ggttcgcgcg	acatttcccc	gaaaagtgcg	acctgacgtc	6900

<210> 3  
 <211> 7038  
 <212> DNA  
 <213> Artificial Sequence

<220>

<220>  
 <223> Description of Artificial Sequence: Construct  
 2C7LBDCS

<400> 3	gatccccctat	ggtagactct	cagtacaatc	tgctctgatg	60
gacggatcgg	gatagatctccc	ctgctccctg	ggaggtcgct	gagtagtgcg	120
ccgcataagtt	aagccagtat	cttgtgtgtt	gcttgaccca	aaagtatctgc	180
cgagcaaaaa	ttaagctaca	ctgctctcgc	atgtacgggc	cgatataacg	240
ttagggtttag	gcgctttttgc	tagtaatacaa	ttacgggggtc	attagttcat	300
gattattgac	tagttatttaa	cttacggtaa	atggcccgcc	ggcctgacgc	360
ttgagttccg	cgttacataaa	atgacgtatg	ttcccatagt	aaacccaata	420
ccgcgccatt	gacgtcaata	tatttacggt	aaactgccca	gggactttcc	480
attgacgtca	atgggttgag	cctattgacg	tcaatgacgg	gctctggcatt	540
atcatatgcc	aagtacgccc	tgggactttc	gtacatctac	gtattagtcg	600
atgccccagta	catgacctta	cggtttttgc	aggtacatcaa	tagcggtttg	660
ctcgtattacc	actcacgggg	ctccacccca	ttgacgtcaa	ttttggcacc	720
actcacgggg	atttccaaagt	aaatgtcgtg	acaaactccg	cccattgacg	780
aaaatcaacg	ggactttcca	gtctataata	gcagagctct	gagccctatg	840
gtaggcgtgt	acggtggggag	atataatcga	ctcactatag	ggagacccca	900
ctgctttactg	gcttatcgaa	ctatggccca	ggcgccctcc	gctggcctagc	960
gtttaaacttt	aagctttagat	tttctaagtc	ggctgatctg	aagcgccata	1020
cgagtctctgc	gatcgccgct	agtgctcgaat	atgcgatcgt	cttgctcagtc	1080
cacaggccag	aagcctttcc	cccacacagg	cgaggaagct	tttgctctgtg	1140
ccttaccacc	cacatccgca	atgaacgcga	gaggcatacc	aaaattccata	1200
gagggaagttt	gccaggagtg	agtcctcgga	tcgcccgttt	tctaagtcgg	1260
gcgcctatgct	tgcccctgtc	caggccagaa	gcccctccag	tgtcgaaata	1320
ggcccatatc	cgctccacca	ttaccaccca	ctcccgccac	cacacagatc	1380
cttcagtcgt	agtgaccacc	ggaagtgttc	caggagtgat	agacgcaaga	1440
tgccgtgag	atttgtggga	actctagaac	tagtagtatt	caaggacata	1500
aatccattta	agacagaggg	gcaccatgga	taaaaacagg	aggaagagct	1560
ctgtccagcc	accaaactagt	aagtggggaat	gatgaaaggt	gggaacgcga	1620
cgcgctccgc	aaatgctaac	acaaagcgcca	ggggtaggta	ggggtagaggt	1680
agggaggaga	atgttgaaac	gagctgccaa	cctttggcca	tgatcaaaag	1740
ggggtctctg	gagacatgga	ccttgtccct	gacggccgac	cgatagctca	1800
ctctaaagaa	aaacgcctcg	ctctatccga	accagacctc	tcagtgagcg	1860
ggagtctgag	cccccatcgc	ctcactcggc	agacagggag	ctggtctaca	1920
ttcgatgatg	ggtctatgta	ttgtggattt	gacacctcat	gatcagctgc	1980
ggcgaaagag	gtgcaggctg	tgatgattgg	ctctcgtctg	cgctccatgt	2040
atgtgctcgt	ctagagatcc	acttgctctt	ggacaggaac	cagggaaaaat	2100
gaagctactg	tttgctcccta	tgctgctggc	tacatcatct	cggttccgca	2160
catggttgag	atctctcgaca	gcttcaaatc	tattattttg	cttaattctg	2220
gcaggagagag	gagtttttgt	agtcctctgga	agagaaggac	catatccacc	2280
atttctgtcc	agacacttga	tcacactgat	ggccaaggca	gtgcctgacc	2340
caagatcaca	gacactttga	tcctcctcat	cctctccacc	atcaggcaca	2400
gcaccagcgc	ctggcccagc	gcatgaaagt	caagaacgtg	gtgcccctct	2460
aggcatgtgag	catctgtaca	acgcctcaca	tgcccccact	agccgtacgc	2520
ctgtggagatg	ctggacgctg	acatgctgcc	ggccgacgcc	ctggacgctc	2580
cctggacgac	ctcgacctgg	tggaagcatt	cgacctggac	atgctgcccg	2640
catgctcgcc	gccgacgccc	cgcagctcta	gagggccctt	tgatcagctc	2700
taagcggcgc	ctcagactca				

ctgattgttc	cagcacaatc	tgttttgcac	ctcccgcgtg	cctcttcctg	ccgtggaagt	2760
tgcactccct	actgtctctt	cttataaaaa	tggagaaatc	ctgactcgat	ctgtgatgag	2820
gtgtcattct	atctctggggg	gtgggggtgg	cgaggaacag	aaggggggagg	atctgggaag	2880
caatagcagc	catctgtggg	atcgtgtggg	ctctatggct	cttgagagcg	aaagaccacg	2940
ctgggggtct	aggggggtatc	ccgcacggcc	ctgtagcggc	gcattaaatg	cgggcggtgt	3000
gtgtgttacc	cgacgcgtga	cctgtacact	tgcacgcgtc	ctagcgcgcg	ctctcttgcg	3060
ttctcttccc	ctctttcttcg	ccaagtctgc	cggtcttccc	cgctacacgc	taaatcgctg	3120
catctcttct	gggttccgat	tatgtgcttt	acgcgaacct	gaccccaaaa	aacttgatta	3180
gggtgatgtg	tccagttagt	ggccatcgcc	ctgtatagacg	gttttttcgc	ctttcgactg	3240
ggatgcacg	ctttttaata	tggaactctt	gttccaaact	ggacaacacg	tcaaccttat	3300
ctcggtctat	ctttttgatt	tataagggat	ttttgggaat	tcgcctcatt	gggttaaaaa	3360
ttagcttgat	taacaaaaat	ttaacgcgtg	ttaatctctg	ggaaatgtgt	tcagttaggt	3420
tgtggaagt	ccccaggctc	ccccaggcag	cagaagtatg	caaaagcatg	atctcaataa	3480
ctgcagcaac	agggttggaa	agttcccagg	ctcccacagc	ggcagaagta	tcaaaagcat	3540
gcattctcaat	tagtgcacaa	ccatagcttc	gcgcctaaact	cgccccaact	cgcccctcaa	3600
ccgccccagt	tccgccattc	ctgcgcccca	tggctgacta	atttttttta	tttatgcaga	3660
ggccagaggc	gcctctcgct	ctcgacttat	cagaagaatg	tggaggaggt	tttttggagg	3720
ctcaggtctt	tgcaaaaagg	ctggcggaag	ttgtatactc	attttggatg	ctgatcacaa	3780
gacaggtagt	ggatcgcttc	gcgatgattg	acaagatgga	tgtcaacgag	gtctctccgc	3840
ctcttgggtg	gagaggctat	tccgtctatc	ctggggcaca	cagacataac	gtgctctctg	3900
tgcgcgcgtg	cttcggctgt	cagcgcgacg	gcgcccggtt	ctttttgtca	agaccacagt	3960
ctccggtgtc	ctgaatgaac	tgcagcagga	ggcagcgcgg	ctatctgtgc	tggccacgac	4020
ggggcttctc	tgcgcagctg	tctgcagcgt	tgtcaactga	cggggaaggg	actggctgtc	4080
attgggggaa	tgcggcggtg	aggatctctc	gtcatctcac	ctgtcctctc	ccgagaagaat	4140
atctcatatc	gctgatgcga	tgcggcgctg	gcatacgctt	ctgcacgcta	cctgccccat	4200
cgacacacaa	cgcaaatcat	ctcatcgagc	agcagctact	cggtatgga	ccggtctgtt	4260
cgatcaggat	gatctggacg	aagagcatca	ggggctcgcg	ccgcacgcac	tgttgcacag	4320
gtcgaaggat	cgcatgcccg	acgcgcagga	ttctcgtctg	acctactggc	atgcctgttt	4380
ggcgaatatc	ctgttggaaa	atggccgctt	ttcttgatct	atcgaactgt	cgccgtgttg	4440
tgtgcgcgac	cggctatgaa	acatagcgtt	ggctaccctg	gatatttctg	aagagcttgg	4500
cggcgcaatg	ctgcacgcgt	tctcgtgtct	tacgcgtatc	cgccctccgc	attcgcaagc	4560
catcgctctc	tatcgctctc	tgtcagatgt	cttctgagcg	ggactcttgg	gttgcaactg	4620
accgaccaa	cgacgcccaa	ctgcctcata	cgagatttgc	atctccaccg	cgctctctat	4680
gaagggtgtg	gcttcggaa	cgttttccgg	gaagcgcggg	ggatgatctc	ccagcgcggc	4740
gatctctatc	tggagtctct	cgcccccccc	aactctgtta	ttgtcactta	taagtgtgat	4800
aaaaataagc	atagatcatc	aaatttcaaa	ataaagaagt	tttttttcat	gcattctagt	4860
tgtggtttgt	ccaaactcat	caatgtatc	tatcatgtct	gtatatacgt	gacctctcag	4920
tagagctctg	cgtaaatcat	ctgtatagct	tttctgtgtg	gaattatgtg	tcocgtcata	4980
atttccacaa	actaacagag	cggaagcata	aagtgtaaag	cttgggggtg	ctaatgagtg	5040
agctaaactc	caattaatgc	ttctgcgtca	ctgcgcctct	tccagtccgg	aaactgtctg	5100
tcgcagctgc	attatgaagt	ggccgaacgc	gcggggagag	cggtgtttgc	gcgacgcgta	5160
tcagctcaact	caaaagcggt	aaatccacgt	tccacagaat	caggggataa	cgaggaaagc	5220
acaatctgag	caaaaaggcca	gcaaaaggcc	aggaacagta	ataaaggcgc	gttgtcgccg	5280
tttttccata	ggctcgcgcc	cctcagcaga	catcacaaaa	atcagacgtc	aagtcaagcg	5340
tggcgaaacc	cgacaggaact	ataaagatca	caggcgtctc	cccgcttga	ctccctcgct	5400
actgtgcgtg	tttgcagact	gccccttacc	ggatcatctg	cgcctcttct	ccctctcgga	5460
actgtgcgtc	gttttgatgt	cgacgcgtgt	aggatataca	tttgtggtga	ggctcgtcgt	5520
ctcagctgtg	ctgtgttgca	cgaaatcccc	gttcagecgc	acctcgtcgc	cttatccgtg	5580
aactatctgc	ttagagtgaa	cgagctcccc	cagcagctat	cgccactgcg	agacagccat	5640
ggttaacagc	tttagacagc	gagatagata	gtgtgtacta	cagagttctt	gaagtggtgg	5700
ctcaatacag	gttcacagag	acagacagta	cttggtactt	gcgctctgtc	gaagcagcat	5760
acottcggaa	gtccactacg	tagctcttga	aaaaaccacg	aaaaaccacg	tggttagcgt	5820
gttttttttt	tttgcagcga	cgagattatc	cgcaaaaaaa	acaacagcta	agaagactct	5880
tgtactcttt	ctacgggggt	gcagctctga	tggaaacaaa	acaacagcta	tgaggtctgt	5940
gtcatgagat	ctatcaaaaa	gcattctcac	tagactcttt	taaaattaaa	ataaggttgt	6000
ataactcaat	aaagtatata	tgtataaact	gtgtctgaca	tgttcaaaat	cttaactcgt	6060
gag						



actcattacc	cattgattcg	cggtttttgg	agacattcaa	tgggcgttgg	tagecgtttg	560
actccagcgg	attcttcagg	ctccaccoca	ttagctccaa	tggagtgttt	ttttggccag	720
aaaatcaacg	ggactttcca	aaatgctcga	acaactccgc	cccattagac	caaatggcag	780
tggaggtctg	acggtgggag	ctctatataa	cgagagctct	ctgctcaact	agagagcaaa	840
ctctcttact	gcttatcgaa	attaatagca	ctccattcat	ggagacagct	ctgtgcctga	900
gttttaacct	aaagtttcga	ccgaagctcg	atccactagt	ccaggttggt	ggaattctcg	960
cgccgcgggg	gattctatca	cgagcgcggc	ctcgagccct	atgcttgccc	tgtcgagctc	1020
tgcgatcgcc	gtttttctaa	tgcgctgtal	ctgaagcgcc	atctccgat	ccacacagac	1080
cagaagcctt	tcaggtgtcg	aaatatgcag	ctgaacttca	gtcgtatgtg	ccaccttaca	1140
accocattac	gcacccacac	agcgcgagag	ctcttttgct	gtgcacattt	tggggagaaag	1200
tttcgcagga	gtgatgaacg	caagagcgat	accaaaatcc	atttaagaca	gagggactcat	1260
agaactagtt	ctctggagaa	catagagagt	gccaaccttt	ggccacagcc	gctcatgtac	1320
aaacgctatt	agaagaaacg	ctctggcctt	tccttcgacg	ccgacacagc	gggtcagctc	1380
ttgttgtagt	ctgcgcccc	catactctat	ctcgagttag	atctccatag	acccttcagt	1440
gaagcttcga	tgatgggctt	actgaccaac	ctggcagaca	ggagagctgt	tcacatgatt	1500
actcggcgca	agaaggggtc	aggctttgtg	gtttgaccc	tcatgatcca	ggctccacct	1560
ctataagatt	ctctgctgta	gattctatgt	attggtctcg	tttggcgctc	catggagcac	1620
ccagggaagc	tactgtttgc	tctaactagt	cttttgaca	ggaaacagct	aaaattgtta	1680
gagggctcag	tggagatctt	cgactgtctg	ctggtcatat	catctcgctt	ccgcattgat	1740
aattctcgag	gagagaggtt	tgtgtgcctc	aaatctatta	tttgtcttaa	ttctggagtg	1800
tacacattct	tgtccagcac	ctctgaagtc	gtggaagaa	aggacccatb	ccaccgtgac	1860
ctggacaaga	tacacagcac	tttgtaccac	ctgtatggca	aggcaggcct	ggcctctgag	1920
cgcagcacac	acggcgtcgc	ccagctctct	ctcatctctc	cccacattag	gcacattagt	1980
acaaagagca	tggagcatct	gtacagctgc	aagtgcagaa	cgctggtgcg	ctctctagtc	2040
ctctgtctcg	agatgctgga	cggccacccg	ctatcatcgc	cccatagccg	tggaaagggca	2100
tcctgtgagg	agaagcgaca	agacacattc	ggcaactcgg	gctctacttc	atcgactctc	2160
ttgcaaaagt	attacatcac	gggggaggca	gaggggttcc	ctgccacagt	ctgtacgcgc	2220
gcgcagcagt	tggagcatct	gcactcgctc	atgctcgccg	ccgacgcctc	ggcagcactt	2280
gaccttgaca	tgctgcgcct	cgacgcactt	gacgaacttg	actggacatc	gctcgccggg	2340
taactaagta	acggcggcgt	cgagctctga	ggggcccttt	aaacccgtct	atcagcctct	2400
actgtgcctt	ctagttcgca	ggcatcttgt	gtttgcctcc	cccctgtgcc	ttccttgacc	2460
ctggaagagc	ctacatccac	tgtctcttcc	taataaaagt	aggaaattgc	atcgactagt	2520
cttgataggt	ctattctctt	tgtgggggtt	gggggtgggg	aggacagcaa	ggggggagat	2580
ttgggaagaca	tacagcgcca	tctgtggggt	cgcggtgggt	ctatggtctc	tggagcgcaa	2640
agaaacagct	ggggtctatg	ggggtctacc	cacgcgcctc	gtgcggcgcg	attaagcgcg	2700
cggggtgtgg	ttgttacgcg	gagactgccc	gctcacattg	ccagcgcctc	agcgccgctg	2760
aatccggcca	tcttcccttc	ctttctcgcc	agcttgcgcg	gcttcccgcg	tcaagctcta	2820
cttgattagg	tcctcttaag	tgtccagttt	agtcgcttac	ggacactcga	cccacaaaaa	2880
ttgacgttgg	gtgatggctt	acgtatggg	ccatcgccct	gatagacgtg	ttttcgccct	2940
taacccattt	gttcagcgtt	ttttaatagt	ggactctgtt	tccaaactgt	aacaaacagc	3000
agtttaaggt	ccggtctatc	ttttgattta	ataaggaatt	tggggatttt	gggctattgg	3060
cttaattagt	agctgattta	ccaggctccc	taacgcaatt	aattctgtgg	ttgtctatgg	3120
ccctcaatgc	tcgaacacac	ctccaggcca	caggcaggca	gaagtataga	agacatgcat	3180
ctcagcagag	cccgagctgc	ttcccaggct	ttcccaggct	cccacaggct	cagaagtatg	3240
tatgcagagg	ccagggcttc	atagttccgc	ccgcctccat	ccctaactcc	gcccactccg	3300
tttggagggc	taggcttttg	gagctatctc	gagctatctc	agaagtatgg	aggagagctt	3360
gatcagagca	caggatgagg	ccggggagct	ccggggagct	gtatattcat	tttcgagtat	3420
ctctccggcg	cttgggtggg	atgatagaac	ggcgagagct	aagatggatt	gcacgagagt	3480
tgctctgatg	cccgctgtgt	ggcgaggggc	ccagaggggc	gctgggtctt	gacaactcgt	3540
accagactgt	ccggtgccc	gaatgaactg	caggaggggc	ctcagctgtg	tttgtgacga	3600
ccgcacagag	cgcttctct	cgagctgtcg	ctcagggggc	ctcagctgtg	atgtgggctg	3660
tggtctgat	tgggcgaa	cgagctgtcg	ctcagggggc	ctcagctgtg	gggaagggag	3720
gagaagaagt	ccatcatggc	gaatgaactg	ctcagggggc	ctcagctgtg	gggagggagc	3780
tgcccatctt	accacaaagc	gaacactcgc	ctcagggggc	ctcagctgtg	gggagggagc	3840
ggtctttctg	atacggatga	ctctggcgac	ctcagggggc	ctcagctgtg	gggagggagc	3900
ttcgcggcgg	tcaagcgcg	gatctctctg	ctcagggggc	ctcagctgtg	gggagggagc	3960
cgctctgtct	cgaaatcat	tgatgcaatg	ctcagggggc	ctcagctgtg		

```

cctctagcta gagcttgagg taatcatggt catagctgtt tcoctgtgtga aattgtatc 4680
cgctcacaa tccacacaa atacgagcgg gaagcataaa gtgtgaaagc tgggggtgct 4740
aatgagctat tcaactcaca ttaattgcgt tgcgctcaat gcccgctttc cagtccggaa 4800
acctgtcggt ccagctcgat taatgaatcg gccacgcgc ggggagagcg ggtttgcggc 4860
gagcggtatc agctcactca aaggcggtaa tacgggtatc cacagataca ggggataacg 4920
caggaagaaa catgtgagca aaaggcgagg aaaaggccag gaaccgtaaa aaggcgcggt 4980
tgctggcggt ttccatagg ctccgcccc ctgacgagca tcacaaaaat cgagctcaa 5040
gtcagaggtg gcgaaacccg acaggagctat aaagatacca ggcgtttccc cctggaagct 5100
ccctcgtagc ctactcctgt cgcagcctgc cgcttacggg atacctgttc gccctttccc 5160
ctctgggaag cgttgcggtt tctcaatgct cagcgtgtag gtatctcagt tcggtgtagg 5220
tcgttcgctc caagctgggc tgtgtgcacg aacccccgt tcaaggccag cgctgcgctc 5280
tatccggtaa ctactgctct gagtccaacc cggtaagaca cgacttatcg ccactggcag 5340
cagccactgt taacaggatt agcagagcga ggtatgtagg ggtgtctaga gatttcttga 5400
agctgtggcg taactcaggg tacactagaa ggacagtatt tgggtatctgc gctctgtcta 5460
agccagtac ctctggaaaa agagtgtgta gctcttgatc cggcaaaaac accacgcgtg 5520
gtagcgggtg ttttttggt tgcaagcagc agattacggc cagaaaaaaa ggatctcaag 5580
aagatccttt gatctcttct acgggggtcg acgctcagtg gaacggaaac ctactgtaag 5640
ggatttttgt catgagatta tcaaaaagga gatccttga gatccttga taactaaaat 5700
gaagttttta atcaatctaa agtatatatc agtaaaacttg gtctgcagcg taaccaatgc 5760
taatcagtag gccacctaac tcagcgatct gtctatttcg tcatccata tgtgcctgac 5820
tccccgtcgt gtatagaact acgatcaggg agggcctacc atctggcccc agtcgtgcaa 5880
tgataccgc agaccaacgc tcaocggctc cagatttatc agcaataccc cagccagcgc 5940
gaaggcgaga gcgcagaagt ggtcctgcaa ctttaccgc ctccactcag tctattaatt 6000
gttgccggga agctagagta agtagttcgc cagttaatag tttgcgcaac gattgtgcca 6060
ttgtctacag catcgttggt tcacgctcgt cggttggtat ggctctatc agtcctgggt 6120
cccaacgctc aaggcgagtt acatgatccc ccatgttgtg caaaaaagcg gttagctcct 6180
tcggctctcc gatcgttgct agaagtaagt tggccgcagt gttatcactc atpgttatgt 6240
cagcactgca taactctctt actgtcactc acctcgtaag atgcttttct gtagctgggt 6300
agtaactaac caagctctc tgagaatagt gtatgcggcg accgagttgc tcttgcggcg 6360
cgtaactacg ggaataaacc gcgcacata gcagaacttt aaaagtgtct atctatgaa 6420
aacgtttctc gggggcgaaa ctctcaagga tcttaccgct gttgagatcc agttcgatgt 6480
aacccactgc tgaccacca tgactctcag catcttttac tttcaccagc gttctcggt 6540
gagcaaaaaa aggaaggcaa aatgcgcgaa aaaagggaaat aaggcgagca cggaaatgtt 6600
gaatactcat actctctctt tttaaatatt attgaaagc tttcagggt tatgtctca 6660
tgagcggata catatttgaa tgtatttaga aaaataaaca aataggggtt ccgcgcacat 6720
ttcccgaaa agtgcacct gacgtc 7746

```

```

<210> 6
<211> 6623
<212> DNA
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence: Construct
C7LBDS

```

```

<400> 6
cagcgatcgg gagatctccc gatccccctat ggtcgactct cagtacaatc tgcctctgatg 60
cgcgatagtt aagccagtat ctgctccctgt ctgtgtgtgt ggaggtcgct gtagtctgcg 120
cgagcaaaat ttaagcatac acgaaggcaag gcttgacggc caattgtcgtt aagaaatcgt 180
ttagggttag gcgttttgcg ctgcttcgcg atgtacggca cagatatacg cgttgacatt 240
gattattgac tagttattaa tagtaaatcaa ttacgggggt atagtttcat agcccatata 300
tggagttccg cgtttacata cttaacgttaa atggcccgcc tggctgcacc cccaacgacc 360
cccgcccatc gacgtcaata atgacgtatg ttcccatagt ttcacgtgca atcgccaata ggaactttcc 420
attgacgtca atgggtgtgac tatttacgggt aaactgcoca ctacttgcca gtacatctac gttattagca 540
atcatatgcc aagtagcggc cctattgacg tcaatgacgg ctacttgcca gttattagca 600
atgcccagta catgacctta tgggaactttc agtacatcaa tggggcgtgga tagcggtttg 660
tcgctattac catggtgatg cggttttggc ctccacccca ttgacgtcaa tgggagttcg 720
acctcagggg atttccaagt cccaccccca acaactccgc cccattgacg caaatggccc 780
aaaatcaacg ggaactttcca aaatgtcgta gtaggctgtg gtaggctgtg agaatggccc 840
gtaggcgtgt acggtgggag gtctatataa ctactatag gtaggaccca gctggtctag 900
ctgcttactg gcttatcgaa attaatacga atccactagt atccactagt cgaattcctg 960
gtttaaactt aagcgttgta cagcctatggt atgcttgccc tgcctagctc 1020
cagcccgggg gatctatggc ccaggcgggc ctgaagcgcc atatccgcat ccacacaggc 1080
tgagcgcgcc gcttttgtaa gtcggctgat

```

cagaagcctt	tccagtgtcg	aatatgcatg	cgtaaactca	gtcgtagtga	ccaccttacc	1140
accacatctc	gcaccacac	agggcagaag	cctttttgct	gtgacatttg	tggggaggaa	1200
ttgtccagga	gtgatgaa	caagaggcat	accaaaatcc	atttaaagaca	gagggactct	1260
agaactagtt	ctgtctggaa	catgagagct	gccaaacttt	ggccaagccc	gctcatgtac	1320
aaacgctcta	agaagcaaac	cctggccttg	ctccgtga	gcaccagat	gctcagtgcc	1380
ttgtttggatg	ctgagccccc	catactctat	tcggagatgt	atctaccag	acccttcagt	1440
gaagcttcta	tgatggcttc	actgaccaac	ctggcagaca	gggagctgg	tcacatgtac	1500
aactggggga	agaggggtgc	agggctttgt	gatttgacc	tccatgatca	ggtccactct	1560
ctagaatgtg	cctggctaga	gatcctgctg	attggctctg	ctctggcgctc	catggagac	1620
ccaggggaagc	tactgtttgc	tcctaaactt	ctcttgga	ggaaaccagg	aaaattgtga	1680
gagggcgctg	tgaggatctt	gcagatgctg	ctggctacat	catctcgggt	ccgcatggat	1740
aatctgcag	gagagggagt	tgtgtgcctc	aaatctatta	ttttgtctaa	ttctggagtg	1800
tcacacattt	ctgtccagac	cctgaagctg	ctggaagaga	aggaccatat	ccaccgagtc	1860
ctggacacata	tcacagacac	tttgatccac	ctgatggcca	aggcaggctc	gaccctgcag	1920
cagcagcacc	agcggtctgg	ccagctcctc	ctcatcctct	cccacatcag	cgacatgagt	1980
aaacaaaggca	tgaggcatct	gtacagcat	aagtgcagaa	acgttggtg	cctctatgat	2040
ctgtgtgtgg	agatgtggga	cgccacccgc	ctacatgcgc	ccactagcgc	tacgcgcgcc	2100
gagcgccctg	aggaacttgc	cctggacatg	acgtccggcg	acgtctgga	cgactcgac	2160
ctgagatctg	tgcggcgga	cgccctggac	gaacttcgac	tggaactgct	cgccgggtaa	2220
ctaaagtacg	ggcgcctcga	gtctagagg	cccggttaaa	cccgctgac	agcctcgact	2280
gtgccttcta	gttgccagcg	atctgttgtt	tgccctccc	ccgtgctctc	cttgaccctg	2340
gaaggtgcca	ctccactgt	cctttcctaa	taaaatgagg	ccgtgtctc	cttgtgtctg	2400
agtagagtgc	attctattct	ggggggtggg	gtggggcagg	gcagcaagg	ggaggattgg	2460
gaagacaata	gcaggcatgc	tggggatg	gtgggctcta	tggcttctga	ggcggaagaa	2520
accagctggg	gctctagggg	gtatccccac	gcgcctctgt	ggcgccgat	aagcgcggcg	2580
gggtgtgtgg	ttacgcgcag	cgtgacgcgt	acacttgcca	gcgcctctag	ccgcgtcct	2640
ttcgctttct	tcctcttctt	ctctgcacg	ttcgccgct	ttcccgctca	agctctaaat	2700
cggggcctac	cctttagggtt	ccgatttagt	gctttacgg	acctgcaccc	caaaaacttt	2760
gattagggtg	atgggttccg	tagtgggcca	tcgccctgat	agacgtgttt	tcgccctttg	2820
acgtttggat	ccacgtttct	taatatgttg	ctcttgttcc	aaactggaa	acactcaca	2880
cctatctcgg	tcatttcttt	tgatttataa	gggattttt	gggatttcg	ctattgttta	2940
aaaaatgagc	tgattttaaca	aaaaattaac	gcgaattaat	ttctgtggat	gtgtgtcagt	3000
taggggtgtg	aaagtcccca	ggctccccag	cagggcagaa	gatgcagaa	catgatctc	3060
aaattagtac	caaccagggt	tgaaagtc	ctcagctccc	catcaggcag	aagtatgaa	3120
agcatgcatc	tcaattagtc	agcaaccata	gtccccccc	taactccg	cattccgcgc	3180
ctaaactcgc	ccagtttcgc	ccattctccg	ccccatggct	tactaatttt	ttttatttat	3240
gcagagcccg	agggcgctct	tgccctcgat	ctattccaga	agttagtgagg	aggtcttttt	3300
ggaggcctag	gctttttgca	aaagctcccg	ggagcttgta	tatccatttt	cggatctgat	3360
caagagacag	gatgaggatg	gttttcgatg	attgaacaag	atggattgca	cgaggtttct	3420
ccggcgcctt	tggtggagag	gctatttcgg	tatgactggg	cacaacagac	cactcgctgc	3480
ttctgatccg	ccgtgttccg	gctgtcagcg	caggggcgcc	cggttctttt	tgtcaagacc	3540
gacctgtccg	tgagccttcc	tgaactcgag	gagaggcgag	cgcgctcttc	gtggctggcc	3600
acgacggcg	ttcccttgcc	agctgtgctc	gacgttgtca	ctgaagcggg	aagggagtgc	3660
ctgctattgg	gcgaagtggc	ggggcaggat	ctcctgtcat	ctcactctgc	tctctccgag	3720
aaagtatcca	tcatgggtga	tgcaatggcg	cggctgcata	cgctgtgac	ggctacctgc	3780
ccatttcgca	accaagcgaa	acatcgatc	gagcagacac	catctcgat	ggagccggct	3840
ctttctgcac	aggatgatct	ggacgaagag	catcaggggc	tcgcgcgac	cgaaactgtc	3900
ggcaggtcca	agggcgcatc	gcccagcgcc	gaggatctcg	tgctgacca	tggcatcgag	3960
tgcttgccga	atatcatgtg	ggaaaaatgg	cgcttttctc	gatcatcga	ctgtggccgg	4020
ctgggtgctg	gcagccctga	tcaggacata	ccgttgacta	cccgatgat	ttgtcaagag	4080
cttgccgcgg	aatgggctga	ccgcttctc	gtgctttta	gtatccgcgc	tcgccgatcg	4140
gcagcgcctc	cctctctatc	cctctttgac	gtgcttctct	gagcgggact	cggggttcg	4200
aaatgaccca	ccaagcgacg	cccaactctg	catcacagga	ttctgatctc	accgcgcgtc	4260
tcctatgaaa	gttgggctct	ggaaatcggt	tcggggagcg	cggtcggatg	atctcccgag	4320
goggggatct	catgctggag	ttcttcgccc	accccaact	gtttattgca	gcttataatg	4380
gttacaaaata	aaagcatagc	atcacaaatt	tcacaaaata	agctattttt	tcactgcact	4440
tttggctggg	tttgtccaaa	ctcatcaatg	tattctatca	gtctgtata	ccgtgcactc	4500
ctagctagag	cttgccgctaa	ctatggctcat	agctgtttcc	ttgtgaaat	tggtatccgc	4560
tcacaaattc	acacaaacata	cgagccggaa	gcataaagtg	taaaagctgg	ttgtgcatac	4620
tggtgagctca	actcacatata	attgcgttgc	gctcactgc	cgctttccag	tcgggaaacc	4680
gactgtgccca	gctgcatttaa	tgaactcgcc	aacgcgcggg	gagaggcgg	ttgcgcgag	4740
cggtatcagc	ctactcaaa	cggttaatac	ggttatccac	agaatcagg	gataccgac	4800
gaaagaaacat	gtgagcaaaa	ggccagcaaa	acgcgcagg	ccgtaaaaa	gcgcgtgtgc	4860
tgccgtttttt	ccataggctc	gcggccctgt	acgagcatca	caaaaatacg	cgctcaagtc	4920
agaggtggcg	aaacccgaca	ggactataaa	gataccagcg	gtttccccct	ggagctcccc	4980
ctggtgcctc	tcctgttccg	accctgcgcg	ttaccggata	cctgtccgcc	ttctccctt	5040
ggcgactcgt	ggcgctttct	caatgcctac	ctgttaggta	ctgtaggtcg		5100

```

tctgcgtccaa gctggggtgt gtgcacgaac ccccggttca gccgcagccg tgcgccttat 5160
ccggtaacta tctgcttgtag tccaaccogg taagacacga cttatcgcca ctggcagcag 5220
ccactggtaa caggatttag agagcgaggt atgtagcggt tgctacagag tctcttgaagt 5280
gggtggcctaa ctacgggttac actagaagga cagtatttgg tatctgcgct ctgctgaagc 5340
cagttacctt cggaaaaaga gttgtagctg cttgatccgg caaacaacac accgctggta 5400
gcgggtggtt ttttggttgc aagcagcaga ttacgcgtag aaaaaaagga tctcaagaag 5460
atcctttgat ctttctctac ggggtctgag ctcagtggaa cgaaaactca cgttaaggga 5520
ttttggtcat gagatctaca aaaaaggatct tcacctagat ccttttaaat taataatgaa 5580
gttttaaatc atctctaaagt atatatgagt aaacttggct tgacagttac caatgcttaa 5640
tcagtgaggc accatactca cgcgctgtgc taccctagat attcatggt gccctgactcc 5700
ccgtcggtga gataactaca atacgggagg ctttaccatc tggcccagct gctgcaatga 5760
taaccgcaga ccaacgctca cgggtccagg atttatcagc aataaacccag ccaagccgaa 5820
ggggcgagcgc cagaagtggt tatccgacct tatccgcctc cgcgcaacgt ttcacatcgt 5880
gcggggaagc tagagtaagt agttcgccag ttgaatagtt tccatcgctg gttgcaattg 5940
ctacaggact cgtgggtgca cgtctgctgt ttggtatggc aaagcggtt agctcctcgc 6000
aacgatcaag gcgagttaca tgatccccc aagcagcgtt atcaactcag gttatggcag 6060
gtcctccgat cgttgctcaga agtaagttgg ccgcagtggt ccgtaagatg cttttctgtg 6120
cactgcataa ttctcttact gtcatgccat cgttaagatg gtagtctctc ttgcccggct 6180
actcaaccaa gtcattctga gaatagtgtg tgcggcgacc gagtctctac attggaaaac 6240
caataccgga taataccgcg ccacatagca gaactttaaa taacctctac tctgattgaa 6300
gttcttcggg gcgaaaaact tcaaggatct taccgtgtgt tccagctggt tgatgtaac 6360
ccactctgcg aaccaactga tcttcagcat cttttacttt ccacagctgt tctgggtgag 6420
caaaaaacag aaggcaaaat gcgcgaaaaa agggaaataag ggcgacacgg aaatgctgaa 6480
taactacact ctctcttttt caatattatt gacaggatga tctgctcatga 6540
gcggatacat atttgaatgt atttagaaaa ataaacaatg aggggttccg cgcacatttc 6600
ccgaaaaagt gccacctgac gtc 6623

```

<210> 7  
 <211> 6818  
 <212> DNA  
 <213> Artificial Sequence

<220>

<220>  
 <223> Description of Artificial Sequence: Construct  
 C7LBDBL

```

<400> 7
gacggatcgg gagatctccc gatccccctat ggtcgactct cagtacaatc tgcctctgat 60
ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg 120
cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caatgcatg aagaactcgc 180
ttaggggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt 240
gatatttgac tagttattaa tagtaatcaa ttacgggggtc attagttcat agcccatata 300
tggagttccg cgttaccata cttaacggtaa atggcccgcg tggctgacgc gccaacgcac 360
cccgccatc gacgtcaata atgacgtgat ttcccatagt aacgcgaata cccaactttcc 420
atcagctca atgggtggag tatttaacggt aaactgccca cttggcagta catcaaatgt 480
atcatatgcc aggtacgcc cctattgacg tcaatgacgg taaatggccc cgcctggcat 540
atgcccagta catggtgatg cgggttttgc ttgaactcaa gtacatctac gtattagtca 600
tcgctattac actcacgggg atttccaagt ctccacccca tgaactcga ttgggcgttg tatggcacc 660
aaatacaacg gaaactttca aaatgtcgtg acaactccgc cagagctctc cctgttgagg ttttggcacc 720
gtaggcgtgt acggtgggag gtctatataa ccagagctct ctagctcga agagatggcg 780
ctgcttactg gcttatcgaa attaatcaga ctcaatagc ttacactatg ggagacccaa cgtcgctacg 900
gtttaaactt aagcttggta ccgagctcgg atccactagt ctagctgtcc tgcagactgc 960
cagccccggg gatcatctgg ccaggcgccc ctgcagccct atgctgtccc tgcagactcc 1020
tgcgatcgcc gctttttcag ctggcgctgat ctgaagcgcc atatccgat ccacacagc 1080
cagaagcctt tccagttgctg aatatgcatg cgtaaactca gtctgagtga ccaacttacc 1140
accacactcc gccaccacac aggcgagaag atcttctgct gtgacattg tgggaggaag 1200
tttgcagaga gtgatgaacg caagaggcat accaaaatcc atcttaagaca gagggaactc 1260
aagaactagt accgagaagg agggagaatg ttgaacacac agtcgcagag agatgattgg 1320
gagggcaggg gtgaagtggg gtctgtcgga gacatgagag ctgccaaact tggggcaagc 1380
ccgctcatga tcaaacgctc taagaagaac agccctggct tgcctcagc gcccgactgc 1440
atggtcagtg ccttgttggg tgctgagccc cccatactct attccgagta tgactctacc 1500
agacacctca gtgaagcttc gatgatgggc ttaactgacca accctgcaga cagggaactg 1560
gttccatcat tcaactgggc gaagagggtg ccaggcttgg tggatttgac cctccatgat 1620
cagggtccac tctagaatg tgctggcta gagatcctga tgattggctc cgtctggcgc 1680

```

tccatggagc	accacaggga	gctactgttt	gctcctaact	tgtccttgga	caggaaccag	1740
ggaaaaatgt	tagaggggat	ggtagagatc	ttagacatgc	tgtgggctac	atcatctcgg	1800
tcccgcatga	tgaatctgca	gggagaggag	tttgtgtgco	tcaaatctat	tattttgctt	1860
aatctctgag	tgtacacatt	tctgtccagc	accctgaagt	ctctgggaaga	gaaggaccat	1920
atccaccagc	tctctggacaa	gatcacagac	actttgatcc	accctgatgc	caaggcaggc	1980
gcagccctgc	agccagcagca	ccagcggctg	gcccgactcc	tcctctatcct	ctccaccatc	2040
aggcacatga	gttaacaaagg	catggagcat	ctgtacagca	tgaagtgcga	gacgttggtg	2100
ccctctctatg	acctgctggt	ggagatgctg	gacgcccacc	gcctacatgc	gcccatcagc	2160
ctctggaggag	catcogtggc	ggagacggac	caaaagcact	tggccactgc	ggcctctact	2220
tcatcgcatc	cccttgcaaaa	gtattacatc	acgggggagg	cagagggttt	ccctgccaca	2280
gtccglacgc	cgccgcagcg	ctcggacagc	tctcagctgg	acatgctggc	ggccgacgcc	2340
ctggagcact	ctgacatgga	gtgctgctgc	gccgacgccc	tggacgactt	cgacctggac	2400
atgctgcggg	gggtataaag	taagcggcgg	ctcgagctca	gagggcccggt	ttaaacccgc	2460
tgtatcgact	cgactgtggc	ttctagtctg	cagcatctct	tgttttgcgc	ctcccccggt	2520
ccctctctga	ccctggaaagg	tggcaactcc	actgtccttt	cctaataaaa	tgaggaaatt	2580
gcactgcatt	gtctagtag	gtgtcattct	attctggggg	gtgggggtggg	gcaggacagc	2640
aagggggagg	attgggaagg	caatagcagg	catgctgggg	atgcgttggg	ctctagctgg	2700
tctgagcggg	aaagaaccag	ctggggctct	agggggtaac	cccacggccc	ctctagcggc	2760
gcattaaagc	cgggcgggtg	ggtggttaac	cgacgctga	ccgctacact	tggccagccc	2820
ctagcgcggc	ctcctttcgc	ttctttccct	ctcctttcgc	ccacgttgcg	ggggctttcc	2880
cgctaacgct	taaatcgggg	catcccttta	gggttccgat	ttagtgtttt	acgggacccc	2940
gaccccaaaa	aaacttgatt	gggtgatggg	tacgtagtgt	ggocactgcc	ctgatagacg	3000
gttttttgcc	tttaacgctt	ggagtccacg	ttcttttaata	gtggactctt	gttcccaact	3060
ggaaacaaac	ctcaaccctt	ctcggtctat	ctcttttgatt	tataaaggat	tttggggatt	3120
toggcctatt	ggtttaaaaa	ttagctgatt	taacaaaaat	ttaacgcgaa	ttaattctgt	3180
ggaatgtgtg	tcagtttaggg	tgtggaaagt	cccaggctcc	cccagcgagg	caagaatgat	3240
caaaagctga	atctcaatata	gtcagcaaac	aggtgtggaa	acgtccccag	ctccccagca	3300
ggcgaatgta	tgcaaaagcat	gcacttcaat	tagtcagcaa	catgatctcc	gcccttaact	3360
ccgcccactcc	cgccctcaac	tcgccccagt	tcgccccatt	ctcgccccc	tggctgacta	3420
atttttttta	tttatgtaga	ggccgaggcc	gcctctgcct	cttagcttat	ccagaagtatg	3480
tgaggaggct	tttttggagg	cttaggcttt	tgcaaaaagg	tcccggaggc	ttgtatatcc	3540
attttcggat	ctgatcaaga	gacaggatga	ggatcgtttc	gcagtatgta	acaagatgaa	3600
ttgcacgcag	gtttctccggc	cgcttgggtg	gagaggctat	ctcgctatga	cgccgcacaa	3660
cagacaatcg	gctgtctctga	tggccgcgtg	tccggctgtc	cagcgagagg	ggccggcggt	3720
cttttttgca	agaccagcct	tccgggtgcc	ctgaatgaac	tgcaggacga	ggcagcggcg	3780
ctactgtgtg	tggccacagc	ggggcttctc	tgcgcagctg	tgtctgcagt	gtcactgaa	3840
gcggggaagg	actggctgct	atggggcgaa	gtgccggggc	aggatctcct	gtcatctcac	3900
cttgctcctg	ccgagaagaat	atccatcatg	gctgatgcga	tgcggcgctc	gcatacgctt	3960
gatccggcta	ccctgccatt	gcaccaccaa	gcgaaacatc	gcacgcagcg	agcacgctat	4020
ccgattgggg	atgctcttgt	cgatcaggat	gatctggagc	gatctggagc	acggcgaggga	4080
ccagccgaac	gtgttcggcg	gctcaaggcg	cgcatgcccg	atgggtggaa	acggcgaggga	4140
accocatggc	gtgttcggcg	gcggaatatc	atgggtggaa	cgctatcagg	atgggtggaa	4200
atcgactgtg	gcgggctggg	tgtggcgagc	gcgtatcagg	gctgacgcct	acatagcgtt	4260
gatatttgct	aagagcttgg	cggcgaatgg	gctgacgcct	tctcgtgctt	tctcgtgctt	4320
gcgcctcccg	atggcagcgt	catcgctctc	tatcgctctc	ttgacagagt	ctcttgaggc	4380
ggactctggg	gttgcaaatg	accgacaagg	cgacggccaa	ctctgcatac	cgatagcttt	4440
attccacgcg	cgctctctat	gaaagggttg	gcttgggaat	cggtttcccg	gacgcgcgct	4500
gtgatgatcc	ccagcggcgg	gatctcaatg	tggagttctt	cgcccaaccc	aactctgtta	4560
tgtcagctta	taattgtttac	aaataaagca	atagcatcac	aaatttccaa	ataaagactc	4620
ttttttcact	gcattctagt	tgtggtttgt	cgtaaatcat	caatgtatct	tatcatgtct	4680
gtataocgt	gaaccttagc	tagagcttgg	acataacgag	gtcatagcat	tttccctgtg	4740
gaaatttgta	tcgcctcaaca	atccacaca	acatacagc	cggaagccta	aagtgtlaag	4800
ctctgggtgc	ctaatgagtg	agctaactca	cattaattgc	gttgcgctca	ctggccgctt	4860
tccagttggg	aaaactgtctg	tggcagctcg	ataatgaat	cgccgcaacg	ctggggagag	4920
gcgggtttgc	gcgagcggta	tcagctcaat	caaaaggcgt	ataacagcta	tccacagaa	4980
caggggatata	cgacggaagg	aaactgtgag	caaaaaggcca	gcaaaaaggc	aggaacccgta	5040
aaaaggccgc	gtttgtggcg	tttttccata	ggctccgccc	ccctgacgag	catcacaaaa	5100
atcgacgctc	aagtcagagg	tggcgaaacc	cgacaggact	ataaagatac	caggcgtctt	5160
ccccctggac	ctccctcgctg	cgctctcctg	ttccgacctc	gcgcgttacc	ggatacctgt	5220
cgccctttct	cccttcggga	agcgttggcg	ttctctcaatg	ctcaocgtgt	aggtatctca	5280
gttcggtgta	ggctcgtcgc	tccaagctgg	gctgtgtgca	cgaaccccc	gtctagccgc	5340
accgctcgcc	cttatccgct	aactatcgct	ttgagtcgaa	cccggtaaga	cagcagctat	5400
cgccactggc	agcagccaat	ggtaacagga	ttagcagagc	gaggttagta	ggcgtgtgta	5460
cagagtctct	gaagttggtg	cctaactcac	gctacactag	aaggacagta	tttggttatct	5520
cgagttcgtg	taagcggctt	accttcggaa	aaagagttgg	tagctcttga	tccggcaaac	5580
aaacaccgcg	tgtgtacggt	ggtttttttt	tttgcaagca	gcagattacg	cgcagaaaaa	5640
aaggatctca	agaagatctc	ttgatctttt	ctacggggtc	tgacgtcag	tggaaacgaa	5700



ccccctctatg	acctgctgct	ggagatgctg	gacgcccacc	gacctacatgc	gcccactagc	2160
cgtacgcgcg	ccgacgcctc	ggagactctc	gacctgggaca	tgctgcccgcg	cgacgcctctg	2220
gacgactctc	actctggacat	gctgcgcgcg	gacgcctctgc	acgactctcga	cctggacatgc	2280
ctcgcggggt	aactaaagtaa	gcgggcgcctc	gagtcctagag	ggcccgcctta	aaccgcgctga	2340
tcagcctcga	ctgtgccttc	tagttgcctac	ccatctgttgc	tttgcctcttc	ccccgtgctc	2400
tccttgacac	tggaaggtgct	cactccccact	gtcccttccct	aaataaaatgc	ggaaatctga	2460
tcgcattgtc	tgagttagtgc	tcattctatt	ctgggggggtg	gggtgggggca	ggacagcaag	2520
gggggaggtt	gggaagacaaa	tagcaggcat	gctggggatgc	cggtggggctg	tatggctctc	2580
gagcgcgaaa	gaaccagctg	gggtctctagg	gggtatcccc	acgcgcctctg	catggcgcca	2640
ttaaagcgcg	cggtgtgtggt	ggttacgcgc	agcgtgacgc	ctacactctgc	cagcgcccta	2700
gcgcgcgcctc	ctctcgtcttt	cttcccttcc	ttctctgccca	gtctgcgcgcg	ctttccccgt	2760
caagctctaaa	atcggggcat	cctcttaggg	ttccgatttta	gtgcttttacg	gcacctcgac	2820
cccacaaaac	tgatgtaggg	tgatgggttca	cgtagtgggc	catgcgccctg	atagacgggtt	2880
tttcgcctct	tgacgttgga	gtccacgttc	tttaaatagtg	gactcttgtt	cccaactcga	2940
acaaactatc	accctatctc	ggtctattct	tttgatttat	aggggatttt	gggaattctgc	3000
gcctatttgt	taaaaaatga	gctgatttaa	caaaaaatga	acgcgaattt	attctgtgga	3060
atgtgtgtca	gttaggtgtgt	ggaaagctccc	caagctcccc	aggcaggagta	aagtatgcgaa	3120
agcatcatctc	taaatagttc	agcaaacagg	tgtggaaaagt	ccccaggctc	cccagcaggc	3180
agaagtatgc	aaagcatcca	tctcaattag	tcagcaacca	tagtcccgcc	cctaactctcg	3240
cccatccgcg	cccctaactc	gcccagttcc	gcccattctc	gcgccattctc	ctgactaaatc	3300
tttttttatt	atgcagaggc	cgaggcgccc	tctgcctctg	agctattcca	gaagttagtga	3360
ggagagcttt	ttggaggcct	aggcttttgc	aaaaagctcc	ggagagctctg	tatatcactt	3420
ctcggattctg	atcaagagac	aggatgagga	tcgtttcgca	tgattgaaca	agatgaattg	3480
cacgcaggtt	ctccgcgcgc	ttgggtggag	aggctattcg	ctcgtatgact	ggacacaagc	3540
acaatcggct	gctctctctgc	cgccgtgttc	cggtgtctag	cgagcggggc	ccccgtttctt	3600
tttgtcaaga	ccgcactgtc	cggtgccctg	aatgaactgc	acgcagaggc	agcgcggcta	3660
tcgtgtgctgc	ccacgacggc	cgttccttgc	gcagctgtgc	ctgcagctgtc	cactgaagcg	3720
ggaaaggact	gcctctctatt	ggcggaagt	cgccggcagg	atctctctgt	atctccactt	3780
cgtcctcgctg	agaaagtata	catcatggtc	gatgcaatgc	ggcggtcgca	tacgtctgat	3840
ccgcctacct	gcccattcga	ccaccaagcg	aaacatcgca	ctacactcgca	acgtctctcg	3900
atggaagcgt	gtcttctcga	tcaggatgat	ctggacaag	agcatcagg	gctcgcgcga	3960
gcgcaactgc	tcgccaggct	caaggcgccg	atgcccgagc	cgcgaggtct	cgctgtgacc	4020
catggcgatg	ctcgtctgct	gaatatcatg	gtggaaaatg	gcgcgttttc	tggtattcatc	4080
tgatgtggcc	ggctgggtgct	ggcggaacgc	tatcaggaca	acgagtgtgc	taccogtgat	4140
attgtcgaa	agcttggcgc	cgaaatgggt	gaccgcttcc	tcgtgtttta	cggtatcgcc	4200
gctcccgatt	cgcagcgctg	cgccctctat	cgccctctgt	acgagtcttc	ctgagcggga	4260
ctctgggggt	cgaaatgacc	gaccaagcga	cgcccaactc	gccatcacga	gatttcgatt	4320
ccaccgcgcg	ctctcatagc	aggttgggtc	tcggaactgt	tttccgggac	gccgcgtgga	4380
tgatctctca	gcgcggggat	ctcatgtctg	agttctctgc	ccaccccaac	ttgtttattg	4440
cttcattaaa	tggtttacaaa	taaaagcaata	gcatacaaaa	tttcaaaaat	aaagcaattt	4500
tttcatctga	ttctagtgtg	ggtttgtcca	aatcatcaaa	tgatatctat	catgtctcta	4560
tacactgcac	agcttggcgt	acacacacaa	aatcatggct	atagctgttt	cctgtgtgaa	4620
atgttatctc	gctcacaaat	taactcaact	tcagagccgc	aagcataaag	tgtaaaagctc	4680
gggggtgccta	atgagttagc	taactcaact	taattgcgtt	gcctctcact	ccccgtttcc	4740
agtcgggggaa	ccctgtctgc	caagctgaat	aatgaatcgc	ccaacgcgcg	gggagaggcg	4800
ggtttgcggc	agcggtatac	gctcatcaa	agggcgtaat	acggttatcc	acagaaatcag	4860
gggaataacgc	aggaagaaac	atgtgagcaa	aaggccacga	taagggccag	aaccgtaaaa	4920
agggcgcggt	gctggcgttt	ttccatagcc	tcgcgcctcc	gcagagcat	acacaaaatc	4980
gcagctcaag	tcagggtggc	cgaaaccgca	caggactata	aagataccag	gcgtttcccc	5040
ctggaaagctc	ctcgtgtgc	tctcctgttc	cgactctgct	gcttaccgga	tactcgtctg	5100
cccttctccc	ttccggaaag	gtggcgcttt	ctcaatgctc	acgctgtagg	tatctcagtt	5160
cgctgttaggt	cgctcctccc	aaagctgggt	gtgtgcacga	ggtaagccac	gacttatcgc	5220
gctgcgcctc	atccgtaaac	tatcgtcttg	agtcacaaac	gtatgtaggc	ggtgctacag	5280
cactcggcag	agccactggt	aaacaggata	cgagagcgag	gacagtattt	ctgtctctgc	5340
agttttgtga	gtggtgctct	aactacgctg	acactagaag	ctcttgatct	ggcaaaaacaa	5400
ctctctgtaa	gccagttacc	ttcggaaaaa	gagttggtag	ctcttgatct	ggcaaaaacaa	5460
ccaccgcctg	tagcgggttgc	ttttttgttt	gcacagcaga	gattacgcgc	agcaaaaaag	5520
gatctcaaga	agatctcttg	atctttttcta	cggtgtctga	cgctcagtg	aacgaaaaact	5580
cacgttaagc	gattttcgtc	atgagattat	caaaaaaggt	cttcacatag	atctttttaa	5640
attaaaaatg	aagtttttaa	tcaacttaaa	gtatatatga	gtaaaactgt	ctcgcagatt	5700
accaatgctt	aatcagttag	gcacctatct	cagcgatctg	gtactcttgc	tcatcatcag	5760
ttgcctgact	ccccgtctgt	tagataacta	cgatacggga	gggcttacc	ctcggcccca	5820
gtgtgcgaat	gataccgcga	gaccacgcgt	caccgctctc	gttaagcagc	tgcaataaac	5880
agccagccgc	aaggggccgag	gcagagaagt	gtcctgcaac	tttatccgc	tccatccagt	5940
ctattaatgt	ttgcggggaa	gctagagttaa	gtagttcgcc	tttaaatagc	ttgcgcgaac	6000
ttgttgccat	tgctacaggc	atcgtgggtg	cacgctctgc	gtttgtgatg	gcttcatcca	6060
gctccgggtt	cccaacgctca	aggcgagtta	catgatcccc	catgttgtgc	gaaaaacggc	6120

ttagctctcctt	cggctctccg	atcggtgtgca	gaagtaagtt	ggccgcagtg	ttatcactca	6180
tggttatgtg	agcactggat	aattctctta	ctgtcatgcc	atccgcgaag	tgcttttctg	6240
tgactgggtga	gtactcaacc	aagtcattct	gagaatagtg	tatgcggcga	ccgagttgct	6300
cttgcccccgc	gtcaataccg	gataataacc	cgccacatag	cagaacttta	aaagtgtcta	6360
tcatttggaaa	gtgtctctcg	ggggcgaana	cttcaaggat	cttaccgctg	ttgagatcca	6420
gttcgatgtga	accactcgtg	gcacccaact	gatctctcag	atcttttact	ttcaccagcg	6480
ttctctgggtg	agcaaaaaaa	ggaaggcгаа	atggccgaaa	aaagggaaat	agggcggaac	6540
ggaaaatgttg	aatcactcata	ctctctcttt	ttcaatatta	ttgaagcatt	tatcaggggt	6600
attgtctcat	gagcgggatc	atatttgaat	gtatttagaa	aaataaaaca	ataggggttc	6660
cgcgcacatt	tccccgaaaa	gtgccacctg	acgtc			6695

<210> 9

<211> 6956

<212> DNA

<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence: Construct  
C7LBDCI

<400> 9

gagcgatggg	gagatctccc	gatccctcat	ggtcgactct	cagtacaatc	tgctctgatg	60
ccgcgatagtt	aaagccagat	ctgctccctg	ctgtgtgtgt	ggaggtcgct	gagtagtgct	120
cgagcaaaatt	ttaaagctaca	acaaggcaag	cgctgaccga	caattgcatg	aagaattctcg	180
ttagggttag	gcgtttttgc	ctgctctcgc	atgtacgggc	cagatatacg	cggttgacatt	240
gatttatgtg	tagttttataa	tagtaataca	ttacggggct	attagttcat	agcccatata	300
ttggagtctcg	cgttacataaa	cttacggtaa	atggcccgcc	tggtctgacc	cccaacgacc	360
ccgcgccatt	gagctcaata	atgacgtatg	ttcccatagt	aacgcccaata	ggcgactttcc	420
attgacgtca	atgggtggac	tatttaccgt	aaactgcaca	cttgccagta	catcaagtgt	480
atcatatgcc	aagtaacgccc	ccatttgacg	tcaatgacgg	taaatggccc	gctctgcatt	540
atgccccagta	catgacacct	tgggactttc	ctacttgcca	gtacatctac	gtattagtca	600
tcgctattac	catgggtgat	cggtttttgc	agtagacata	tgggcgtgga	tagcgcttctg	660
actcacgggg	atttccaagt	ctccaccccc	ttgacgtcaa	tgggagtgtg	ttttggcacc	720
aaaactcaac	ggacttttcca	aaatgtcgta	acaaactcgc	cccattgacg	caaatggcgg	780
gtaggcgctg	acggtggggag	gtctatataa	gcagagctct	ctggctaact	agagaaacca	840
ctgcttactg	gcttatgcga	atttaatacga	ctcactatag	ggagacccaa	gctggctagc	900
gtttaaacct	aagctttgta	ccgagctcgg	atccactagt	ccagttgtgt	ggaattctcg	960
cagcccgggg	gatctatggc	ccaggcgggc	ctcgagccct	atgcttgccc	tgtcgagctc	1020
tgcgatcgcc	gctttttctaa	gtcggtgatg	ctgaagcgcc	atatccgcac	ccacacaggc	1080
cagaagcctt	tcaggtgtcg	aaatagcatg	cgttaactta	gtcgtagtga	ccactttacc	1140
accacacatc	gcaccacacac	agggcgagaag	ccttttgctc	gtgacatttg	tgggagggaag	1200
tttggcaggga	gtgagtgaac	caagaggcat	acccaaatcc	atttaagaca	gaggaactct	1260
agaactagtga	gtatctcaagg	acataaacgac	tatatgtgtc	ccgcacacaa	ccagttgcacc	1320
attgatataaa	acaggagagaa	gagctgcccag	gcctgcggcg	tacgaacagt	ctcacagagc	1380
ggaatgatga	aaggtgggat	acgaaaagac	cgaagaggag	ggagaatggt	gaacacacag	1440
gcgcacagag	atgatgggga	gggcaagggt	gaagtggggg	ctgctggaga	catgagacagt	1500
cggcaagcctt	ggccaagccc	gctcatgalc	aaacgctcta	agaagaaacg	ctctggcctgt	1560
tccttgacgg	ccgaccagat	gtgcagtgcc	ttgttgtagt	ctgagccccc	catactctat	1620
tcgaagtatg	atccctacag	acccttcagt	gaagcttcga	tgatggggct	acgacaccaa	1680
ctggcgacaga	gggagctagt	tcacatgatc	aactggcgca	agaggggtgc	agcctttgtg	1740
gatttgacc	tcctatgatca	ggctcacctt	ctgagattgt	ctctgctaga	gatctctgat	1800
atttggtctcg	ctggcgctgc	catggagcac	ccagggaagc	tactgttttg	tcctaactgt	1860
ctcttgagca	ggaaccaggg	aaaatgtgta	gagggcatgg	ttggagattc	cgacatgctg	1920
ctggctcatca	catctcgggt	ccgcgatgat	aatctgcagg	gagagagagt	ttgtgtccctc	1980
aaatctatta	ttttgcttaa	ttctggagtg	tcacactttc	tgctccagca	ctcgaagtct	2040
ctgggaagaga	aggacccatt	ccaccgagtc	ctggacaaga	tcacagacac	tttgatccca	2100
ctgatgtgcca	aggcaggcct	gaccctgcag	cagcagcacc	agcggctggc	ccagctctct	2160
ctcatctctc	cccatcatcgc	gcacatgagt	aacaaaggca	tggaagcatc	gtacagcatg	2220
aagtgcaaga	acgtggtcag	ctctctatgc	ctgctctcgg	agatgctgga	gcgccacgcg	2280
ctgactcggc	ccactagctc	tggaagggga	tcctgtggag	agacggacca	agaacctatt	2340
gccactcggg	gctctacttc	atcgcatctc	ttgcataaag	attacatcac	gggggaggca	2400
gaggggtttcc	ctggcacagt	ccgtacgcgc	gcgcagccgc	tggaagcatt	cgacotggac	2460
atgctcggcg	ccgacgcctc	ggacgacttc	gaactggaca	ctgctcggcg	ccagccctgt	2520
gacgactctg	acctggacat	gctgcggggg	taactaagta	agcggccgct	cgagctcaga	2580
ggggcccgctt	aaaccgcgtg	atcagcctcg	actgtgcctt	ctagttgcca	gccatctgtt	2640

gtttgcccct	cccccggtgcc	ttcccttgacc	ctggaagggtg	ccactcccac	tgtcctttcc	2700
taataaaatg	aggaaatctgc	atcgcatctgt	ctgagtaggtg	gtcattctat	tctgggggggt	2760
gggttggggg	aggagacagca	gggggaggatg	tggaagagca	atagcagcga	tgtctggggat	2820
gcggtgggct	ctatgggtctc	tgaggcggaag	agaaccagctg	ggggctctag	gggggtatccc	2880
cacgcgcctc	gttagcggcgc	atlaagcgcgc	gcgggtgtggt	tggttacgcgc	cagcgtgcacc	2940
gctcacacttg	ccagcgccctc	agcgcccgctc	ccctttcgctt	ttctccccttc	ctttctcgccg	3000
acgttcgcgc	gcttctcccg	tcaagctctca	aatcgggggca	ttccctttagg	gttccgattt	3060
agtgctttac	ggcaactcga	ccccaaaaaa	cttgattagg	gtgatggttc	acgttagtggg	3120
ccatcgccct	gatagacggtg	ttttcgccctc	ttgacgttgg	agtccacgtt	cttttaaatgt	3180
ggactcttgt	ttccaaactcg	aacaacactc	aaactctatct	cggtctctatc	ctttgaattta	3240
taaggagattt	ttggggatttc	ggcctatttgg	ttaaaaaatg	actgtgattta	acaaaaattt	3300
aacgcgaatt	aattcttgtgg	aatgtgtgtg	agtttaggttg	tggaagtcgc	ccagggtccc	3360
caggcaggcca	gaagtatgcga	aagcatgcgat	ctcaattagt	cagcaaacag	gttgaggaaag	3420
tcccaggatc	ccccagcagg	cagaagtatc	caaaagcatcg	atctcaatta	tgcagcaacc	3480
atagtcccg	ccctaactcc	gcccaccccg	ccctaactc	cgcccagctt	cgccattctc	3540
cgcccccact	gctgactact	tttttttatt	tatgcagagg	cgaggcgccg	ctgtcctctc	3600
gagctattcc	agaagttagtg	aggaggctttt	tttgagggcc	taggtctttg	caaaaagctc	3660
ccggggagctt	gtatatccat	tttcggatct	gatcaagaga	caggtagtagg	actgtctgcg	3720
atgattgaac	aagatggattt	gcacgcaggtg	tctccggccg	cttgggttga	caggctatttc	3780
ggctatgact	gggcagaaca	gacaatcggg	tgctctgatg	ccgctgtgtg	cgcgctgtca	3840
cgcgaggggc	gcccgggtctt	ttttgtcaag	accgaactgt	ccggtccctc	gaatgaactg	3900
caggcagagg	cagcgccgctc	atcgtggctg	gccaacggc	cgcttccctg	cgagctgtgtg	3960
ctcgacgcttg	tcaactgaagc	gggaaggagc	tggtctgcat	ttggcgcaagt	gcggggggcag	4020
gatctcctgt	catctcacct	tgctcctgcc	gagaaaagat	ccatcatggc	tgatgcaatg	4080
cgccgctgtc	atacgtctga	tccggctacc	tgcccattcg	accacaacgc	gaacatctcg	4140
atggtcagtc	cagcttaoctg	gatgggaagc	gctcttgttc	atcaggatga	cttggacgaa	4200
gagcatcagg	ggctcgcgcg	agccgaaactg	ttcccgccg	tcaaggccgc	catgcccagc	4260
ggcgaggatc	tcgtctgtgac	ccatggcgat	gcctgtctgc	cgaaatcatc	ggttgaaaaat	4320
ggccgctttt	ctggattcat	cgaactgtggc	cggtctgggtg	tgccggaagg	ctatcaggaa	4380
atagcgtttt	ctaccocgtga	tattgtctgaa	gagctttggcg	gcgaatggcg	tgaccgcctt	4440
ctcgtgtctt	acggtatcgc	cgctcccgat	tcgcagcgca	cgcccttcta	tgcctcttct	4500
gcagagattct	tctgagcggc	actctggggt	tcgaaatgac	tcgacaacgc	agcccaacc	4560
tttccatcag	agattttcgat	ttccaccgccc	ccctctatga	aaggttggcg	ttccggaatcg	4620
ttttcccgcc	cgcggcgctgg	atgatcctcc	agccgcggga	ttctcatgtg	gagttctctc	4680
ccccccaaaa	cttgtttattt	gcagcttata	atgtttacaa	ataaagcaat	agcatcacaa	4740
attttccaaa	taaaagctattt	ttttcactgc	attctagtgt	tggtttgtcc	aaactcatca	4800
atgtatcttta	tcatgtctctg	ataccgtcga	ccctcatgta	gagcttggcg	taatcatgtg	4860
catagctggtt	tctctgttga	aattgttatc	cgctcacaa	ttccacacac	atcacgagcg	4920
ggagcatcaa	gtgtaaaagc	tggggtgcct	aatgagtgag	ctaactcaca	ttaatgtcgt	4980
taogctatac	gcgccgtcttc	cagtcgggaa	acctgtcgtg	ccagctcatc	taatgaatcg	5040
gccaacgcgc	ggggagagtc	gggttgcggc	gagcggtatc	agctcactca	aaaggcgtaa	5100
tacggttatc	cacagaatgc	ggggataacg	caggaaaagaa	catgtgagca	aaaggccagc	5160
aaaaggcgag	gaaccgtaaa	aaggccgcgt	tgtccgcgtt	tttccatag	ctccgcccc	5220
ctgacgagca	tcacaaaaat	cgaagctcaa	gtcagagggtg	gcgaaaaccg	acaggatact	5280
aaagatacca	ggcgctttccc	ccctggaagct	ccctcgctgc	ctctcctgtt	cgaccctcgt	5340
cgcttacgcg	ataactctcc	gctctttctc	ctctgggaag	ctgtggcgct	tctcaatgct	5400
cacgctgtag	gtatctcagt	ctggtgtagg	ctgttctgct	caagctgggg	tggtgtcacg	5460
aacccccctc	tcagcccgacg	cgctggcgct	taacgggtaa	ctatcgtctc	gagttccaacc	5520
cggtaaagca	cgaacttatcg	ccactggcag	cagccactgg	atacaggatt	agcagataga	5580
ggatgttagg	cggtgctaca	gagttcttga	agtggtggcc	taactacggg	tacactagaa	5640
ggactatgatt	tggttatcgc	gctctgctga	agccagttac	ctgtgaaaaa	agagtttgta	5700
gctcttgatc	cgccaaaaaa	accaccgctg	gtagccgttg	tttttttgtt	tgcaagcgac	5760
agattacgcg	cagaaaaaaa	ggatctcaag	aagatcctt	gatctttctc	accgggtctg	5820
acgttcacgtg	gaacgaaaaa	tcacgttaa	ggattttgtg	catgagatta	tcaaaaagga	5880
tctcaactca	gatcctttta	aattaaaaat	gaagttttaa	atcaactcaa	agctatatgt	5940
agtaaaattg	gtctgacagt	taccaatgct	taatcagtg	ggcacctcat	tcagcagctc	6000
gtctatttctg	ttcatccata	gttgcttgac	ttcccgctgt	gtagataaact	acgatacggg	6060
agggtctaac	atctggccccc	agtgctgcaa	tgataccgcg	agacccaacg	tcaccggctc	6120
cagatttatc	agcaataaac	cagccagcgcc	gaaggccgga	gcgcagaagt	ggtctcgcaa	6180
ctttatacgc	ctccatccag	tctattaaat	gttgccggga	catgagagta	agtagttcgc	6240
cagtttaatg	tttgcgcaac	gttgttgcca	ttgctacagg	ctacgttggtg	tcacgctcgt	6300
ctgtttggtat	ggctctaatc	agctccgggt	ccccacgatc	aaggcgagtt	acatgatccc	6360
cagtttgtgtg	caaaaaagcg	gttagctcct	tcggtctctc	gatcgtgtgc	aaaggttaag	6420
tgggccgcagt	gtttatcactc	atggttatgt	cagcactgca	taattctctt	actgtctatc	6480
catccgttaag	atgctttttct	gtgactgggt	agtactcaac	caagtcattg	tgagaaatgt	6540
gtatcgccgc	accagattgc	tcttgcccg	cgtaataacg	ggataataac	gcgccacata	6600
gcagaaacttt	aaaagtgtctc	atcattggaa	aacgtttctc	ggggcgagaa	ctctcaagga	6660



tctttaccgc	acctgcgc	caaaaacgt	gtatagggtg	atggtttcacg	tagtgagcca	3000
tgcctcgat	agacggttt	tgcgctttg	acgttggaat	ccacgctttc	taattaggtg	3060
tctctgttc	aaacttgaa	aaactcaac	ctcatctcgg	tctatttttc	tgattttaaa	3120
gggatttttg	ggatttcgc	ctattgttta	aaaaatgacg	tgattttaaa	aaaaatttaa	3180
ggcaattgat	tctgttgaa	gtgtgtcagt	tagggttgtg	aaagtcccca	ggctccccag	3240
gcaggcaga	gtatgcaca	catgcacat	aaattgtcag	caaccagggt	tggaaagtgc	3300
ccaggctccc	cagcaggcag	aagtatgcga	agcatgcgat	ctaatagtcg	agcaaaccta	3360
tgcctgcgcc	taactccgcg	catcccgccc	ctaaactccg	ccagtctcgc	ctattctccg	3420
ccccatgcgt	gactaatttt	ttttattttt	cgagagcccg	aggccgccta	ggctctcgag	3480
ctattccaga	agtagttgag	aggctttttt	ggaggctcag	gcttttgcca	aaagctccgc	3540
ggagcttgtt	ctatcatttt	cgagctctat	caagagacag	gagtggagtc	gtttcccgat	3600
attgaacaa	attgattgca	ccgaggtttg	ccgcgcctct	gggtggagag	gctattccgc	3660
tatgatcggg	caacacacag	aatccgctgt	tctgatcccg	ccgtgtttccg	gtcttcacgg	3720
gagggggccc	ccgtctcttt	tctgaagac	gacctgtccg	tgtccctgaa	tgaattcgag	3780
gacgagtgac	ccgcgctatc	gtgtcgtgcc	acgacggggc	tctcttccgc	agctgtgtct	3840
gcagcttgca	ctgaagcggg	aaaggagctg	tctgtatttg	gcgaagtggc	ggggcaggat	3900
ctctgtcatt	ctcacctctg	tcttcccgag	aaagtattca	tctctgctga	tgcattgtcg	3960
ccgtctcata	ccgtctgatc	ggctacactg	ccattcgacc	accaagcgaa	acatcgcatc	4020
gagcagacac	gtactccgat	ggaagccggg	tctgtgcata	aggatgatct	ggagcgaagag	4080
catcaggggc	tgcgcgcacg	cgaaactgtc	gccagctgtc	aggccgcctg	gcccgacgcr	4140
gaggtatctg	tcgtaaccca	tggcgatgcc	tgtcttcgca	atatcatgtg	ggaaaactgc	4200
ggcctttctg	gattcatcga	tctgtggccg	tctgggtgtg	ggcagccctg	tcaggacata	4260
gtgttttgca	cccgctgatc	tgtctgaagag	tcttggccgg	aattggctgc	ccgctctctc	4320
gtcttttagc	gtatccgcgc	tcccgattcg	cagcgcatcg	cccttcgtca	ctctcttgac	4380
gagtttctct	gagcgggaact	tctggggttg	aaatgaccga	ccaagcgagc	cccaacctgc	4440
ctcacgagca	tcttgatttc	accgcgcctc	tctatgaa	tgtgggcttc	ggaaactgtt	4500
tccgggcagc	ccggctggatg	atctcccacg	cgccgggaat	catctgggag	tgtctgcgcc	4560
accccaactc	gttttatgca	gtctataatt	gttacaata	aagaaatgct	atcacaaatt	4620
tcacaaatat	agcatttttt	tcaactgatt	ctagtgtgtg	tttgtccaaa	ctcatcaatt	4680
tatcttatca	tcttgtata	ccgtcgatcg	ctagctagag	cttggtcgta	tcatggtcat	4740
agctgttttc	tgttgtgaa	gtttatccgc	tccaaacttc	acacacata	cgagccggaa	4800
gcataaagtg	taaaagctcg	gtgtccatct	gagtgagcta	actcacatta	attcggcttg	4860
ctgcactcgc	cgctttccag	gtgcgaaac	tgtcgtcgca	gctgcattat	tgaactcgcc	4920
aacgcgcggg	gagaggtccg	tctggcgccc	cggtatcagc	tcaactaaag	ggcgtaatac	4980
ggttatccac	agaatcaggg	gataacagct	gaagaacatc	gtgagcaaaa	ggccgacaaa	5040
agccaggcaa	cgctaaaaag	cgccgcttgc	tggcgttttt	ccatagagtt	gcgccccctg	5100
acgagcatca	caaaaatcga	cgctcaagtc	aggaggtggc	aaaccccgca	ggactacata	5160
gtacaccagg	gttttccccct	ggaagctctc	togtgcgtct	tccgtttccg	acctcgcgcg	5220
tatccggata	ccgtgcgcgc	tttctccctt	cgggaaagct	ggcgctttct	catgtctcac	5280
gtgttagtta	tctcagttcg	tgttagtgct	tctgcctcaa	gctgggctgt	gtgcacgaac	5340
ccccctgtca	ggccgacggc	tgcgctctc	ccggttaact	tgcgtctgag	tccaacagcg	5400
taagacgca	cttatccgca	tctggcacat	ccactggtaa	caggattgat	agaacggagt	5460
tgtatggcg	tatctcgcg	ctcttgaagt	gggtgacctt	ctacggctac	actagaagga	5520
cttgatcgct	tgatcgcgct	cccgctggta	cagttacctt	cggaaaaaga	gttgttagat	5580
cttgcgcgag	aaaaaaaacg	ccggtgggtt	ccggtgggtt	tttttgttgc	aagcagcaga	5640
cttagcgccg	caaaaacagg	ctctgaagga	atctcttgat	ttttcttagc	gggtctgacg	5700
ctcaatggcg	gcaaaactga	tgaataaggta	ttttgtcatc	gagattatca	aaaagagctc	5760
taacctctgt	ctcttttaaa	gttttaatac	gttttaatac	atactaaagt	atatatgatt	5820
tattccgttc	gttcacattg	cagtgaggcc	acacatctca	acacatctca	gcgatctgtc	5880
gctttaccac	tgtggccagt	ctacgtctta	ccacgtctca	ataccgggag	ataccgggag	5940
attatcacgc	tggcccacgt	tacggcgaga	ggggcgagcg	cagaagtgtg	ccggtcactg	6000
tctcgcctct	catcacagtc	ccacgcggaa	gcggggaagc	tagagatggt	agttccctgc	6060
tcttatagtt	ggcgaacggt	gttccgattg	ctacaggcat	ggctgtgtca	cgctcgtctg	6120
ttgttatggt	tctcatcagc	agctcgtctc	aacgatcaag	gcgagtatac	tgtatcccca	6180
tggttgtgca	aaaaagcggt	agctcccttc	gtctccgat	cggttgtaga	agtaagatgt	6240
ccctcagttg	attactcatg	gttatggcag	catcgtcata	tctctctact	gtcatccgat	6300
ccgtgaagtg	ttctttctgt	actcggtagt				

<211> 6567  
 <212> DNA  
 <213> Artificial Sequence

<220>

<220>  
 <223> Description of Artificial Sequence: Construct  
 E2CLBDAS

<400> 11

gacggatcgg	gagatctccc	gatccccc	ggtcgactct	cagtacaatc	tgctctgatg	60
ccgcatagtt	aagccagtat	ctgctccctg	ctgtgtgtgt	ggaggtcgct	gagtagtgctg	120
cgagcaaaat	ttaagctaca	acaaggcaag	caattgcaga	taagtaactg	taagtaactg	180
ttagggttag	cgctttttcg	ctgcttcgcg	atgtacgggc	catagatacg	cgcttgacatt	240
gattattgac	tagttattaa	tagtaatcaa	ttacggggct	attagttcat	agcccatata	300
tgtagttccg	cgttacataa	cttacggtaa	atggcccggc	tggtcagacc	cccaaccgacc	360
cccggccatt	gacgtcaata	atgacgtatg	ttcccatagt	aacgccaata	ggcaattctcc	420
attgacgtca	atgggtggag	tattttacgg	aaactgccca	cttggcagta	catcaaatgtg	480
atcatatgcc	aagtagccgc	ccatttgacg	tcaatgacgg	taaatggccc	gctggcgcat	540
atgcccagta	catgacotta	tgggactttc	ctacttgcca	gtacatctac	gtattagtaca	600
tcgctattac	catggttgatg	cggtttttgg	agtacatcaa	tgggcggtga	tcagcgctttg	660
actcacgggg	atttccaagt	ctccacccca	ttgacgtcaa	tgggagtttg	ttttggcacc	720
aaaatacaac	ggactttcca	aaatgtcgta	acaactccgc	cccattgacg	caaatggggcg	780
gtaggcgctg	acgggtgggg	gtctatataa	gcagagctct	ctggcttaac	agagaaccca	840
ctgcttactg	gcttatcgaa	attaatacga	ctcactatag	ggagaccacca	gctggctagc	900
gtttaaactt	aagcttagat	ctatggccca	ggcgccctct	gagcccgggg	agaagcccta	960
tgcttgcctg	gaatgttgta	agtcctcttc	ctagagctct	caactgggtg	gccaccagcg	1020
taccacacag	ggtagaaaaa	cgataataat	cccagagtgc	ggcaaatctt	tttagtgactg	1080
ccgcagcatt	gctgcaccac	aacgcactca	tactggcgag	aacccaatac	aatgtccaga	1140
atattggcag	cttttcagcc	gctctgacaa	gctgggtcgt	caccacaact	ctcacaccgg	1200
taaaaaaact	agttctgtcg	gagacatgag	agctggccaa	ctttggccaa	cccgcctcat	1260
gatcaaacgc	ctctaagaag	acagcctggc	cttgtccctg	acggcgccac	agatgggtcag	1320
tgctcttgtg	gatgctgagc	ccccatact	ctattccgag	tatgatctca	ccagaccctt	1380
cagtgaagct	tcgatgatgg	gcttactgac	caacctggca	gacaggggag	tgattccaat	1440
gatcaactgg	gcgaagaagg	tgccaggctt	tgtggatttg	accctccatg	atcaggtcca	1500
cccttctaga	tgtgcctggc	tagagatcct	gatgattggg	ctcgtctcgg	gctccatgga	1560
gcaccaccgg	aagctactgt	ttgctcctaa	cttgcctctg	gacaggaacc	agggaaaaatg	1620
tgtagagggc	atggtggaga	ctctcgacat	gctgctggct	acatcatctc	gggtccgcatt	1680
gatgaactcg	atggtggaga	agtttgtgtg	ctcctaaact	attatttttg	tttaattctgg	1740
agtgtacaca	tttctgtcca	gcacccctga	gtctctggaa	gagaaggacc	atatccaccg	1800
agtcctggac	aagatcacag	acactttgat	ccacctgatg	gccaaaggcg	gctgaccctt	1860
gcagcagcag	caccagcgcc	tgggccagct	ctctcccatc	ctctccccca	tcaggcacat	1920
gagttaacaaa	ggcatggagc	atctgtacag	catgaagtgc	aagaacgtgg	tgccctctta	1980
tgacctgcgt	ctggagatgc	tggaagcccca	cgcgctacat	gcgcacacat	tcgcagaccg	2040
gcgcagacgc	ctggatgggc	ctgacccggga	catgctgcgc	cgccagccct	ggtagcagatt	2100
cgaactggag	atgctgcgcg	ctgacgcgct	ggagcgtctc	gacctgggaca	tgctgcgggg	2160
gaactcaagt	aagcgccgct	ctcgagctag	agggcccggt	taaacccgct	ctgacgcctc	2220
gactgtgcct	ctagtgtgoc	agccatctgt	tgcttgcgcc	tcgcccgctg	cttccttgac	2280
ctctggaagt	ggcaatccca	ctgtcccttc	ctaataaaat	gagggaattg	catcgcatgg	2340
tcgtagtagt	tgtaattcta	ttctgggggg	tggggtgggg	caggtaggga	agggggaggga	2400
ttgggaagac	aatagcagcc	atgctggggg	tgccgtgggc	tgatagctgc	ctgaggggga	2460
aagaacacac	tggggctcta	gggggtatcc	ccacgcgcc	tgtagcgccg	cattaagcgc	2520
ggcggtgtgt	gtggtttacg	gcagcgtgac	cgctacactt	gccagcgccc	tagcgcccg	2580
tcctttcgct	ttctccctct	cccttctcgc	acagttcgcc	ggctttcccc	gtcaagctct	2640
aaatcggggc	atcccttttg	ggttccgatt	tagtgtttta	cgccacactc	accccaaaaa	2700
actctgattg	gggtgatggt	cacgtagtgg	gccatcgccc	tgatagtcag	ttttcgccc	2760
tttgacgttg	gagtcacagt	ctctttaatg	tggaactctg	ttcccaactg	ggcaacacat	2820
caaccctatc	tcggtctatt	cttttgattt	ataagggatt	ttggggattt	cgccctattg	2880
gttaaaaaat	gagctgaatt	aaacaaaaat	taacgcgaat	taattctgtg	gaatgtgtgt	2940
cagtttaggt	gtggagtgct	cccaggctcc	ccaggcaggg	agaagtatgc	aaagcatgca	3000
ttcctaattg	tcagcaacca	gggtgtgaaa	gtccccaggg	ccccagcagc	gcagaagtat	3060
gcgaacgatg	catctcaatt	agtcagcaac	catagctccg	ctcccaactc	cgcccatccc	3120
gcgccctaac	ccggccagtt	ccggccattc	tcggcccaat	ggctgactaa	ttttttttat	3180
ttatcgagcg	gcggagggcg	ccctctgcct	tgagcttatt	cagaagtagt	gagggagctt	3240
ttttgggggc	ctaggctttt	gcacaaaagt	ccggggagct	tgtatatcca	ttttcggtac	3300
tgatcaagag	acaggatgag	gatcgtttcg	catgattgaa	caagatggat	tgcaacgagg	3360

```

ttctccggcc gcttgggtgg agaggctatt cggctatgac tggggcacaac agacaaatcgg 3420
ctgctctgat gccgcgtgtg tccggctgctc agcgcagggg cgcccggttc ttcttgtcaa 3480
gaccgacctg tccggtggcc tgaatgaact gcaggacgag gcagcgcggc tatcggtggc 3540
ggccacgacg ggcgttccct gcgcagctgt gctcgacgtt gtcactgaag cgggaagagg 3600
ctgggtctgta ttgggcgaag tgccggggca ggaatctctg tatctctcgc tctctcctgc 3660
cgagaaagta tccatcatgg ctgatgcaat gcggcggtcg catcgacttc ggcggaagc 3720
ctgcaccttc gaaccacaag atctggagca agagcatcag gggctcgcg cagcgaactc 3780
cggtctgtgc gatcaggatg gcatgcgca cggcgaggat ctgctgggca ccgatggcga 3840
gttcccaagg ctcaagcgcg tgggtggaaa tggcgctgtt tctggactca tgcactgtgg 3900
tgccgtcttg gtcggcgacc gctatcagga ctatagcgtt gctaccgctg atattgtctga 4020
agagcttgcc ggcgaatggc ctgacgcgtt cctcgtgctt tgcagagtcc ttctgagcgg gactctgggg 4140
ttcgacagcg atcgctcttc atcgcccttc tgccacccac ctgcactcac gagatttcga ttccaccgcc 4200
tccaaatgca cgcagcaaac gacgcccac gcttccggg acgcccggct gatgatcttc 4260
gcctcttatg aaagctgtgg cttcggaatc gtagttcttc aaatgtatct atcattgtct 4320
cagcgcgggg atctcatgct tagcatcaca aatttcacaa ataaagcatt tttttcaact 4380
aatgtgtaca ataaagcaca taactcatgc caaactcact atcattgtct ttaaccgtgc 4440
cattctacat agagcttggc gtaatcatgg tcatagctgt ttctctgtgt aaattgttat 4500
ccgctcagca ttccacacaa catacagacc ggaagcataa agtgtaaaag ctgggggtgc 4560
taatgagtga gctaactcac attaatgtgc ttgcgctcac tgcccgtct ccaagtccgg 4620
aaactgtcgt atctcatgct ttaatgaatc ggccaaacgc cgggcgaggg cggtttgcgg 4680
cgagcgggat cagctcactc aaaggcggta atacggttat ccacagatac aggggataac 4740
cgaggaagaa acatgtgagc aaaaggccag caaaagccca cctgacgagc atccaaaaaa tcgacgtctc 4800
ttgctggcgt ttttccatag gctccgcccc gacaggacta ccgcttaccg gatacctgtc cgtctttctc 4860
agtcagaggt ggccgaaccc gcgcacccgt tctcctcaatg ttgatctcag ttccgtgtag 4920
tcctctgtgc gctctcctgt tccgacccgt cggcttaccg gtaactgtct tccgtttctc 4980
ccttcgggaa gctgtggcgt ttctcaatgc ctgacgtgta taacgcttat tccgtgtgca 5040
gtcgttcgct ccaagctggt ctgtgtgcac gaaccccccg ttaacgcgca cgcctgcgcc 5100
ttatccctga actatgctct tgagtccaac ccggtaaagc acgactttac gcgaactgca 5160
gcagccactg gtaaccagat tagcagagcg aggtatgtag gcggtgtctc agagtcttg 5220
aagtgtgtgg ctactacgg ctacactaga aggcacagt attggtatctg gctctgtgct 5280
aggccagtta ccttcggaaa aagagtttgt agctcttgat ccggcaaaac aaccaccgt 5340
ggtagcgtgt gtttttttgt ttgcaagcag cagattacgc gcagaaaaaa aggatctcaa 5400
gaagatcctt tgatcttttc tacggggctc gacgtcagt ggaacgaaaa ctacagttaa 5460
gggattttgg tcatgagatt atcaaaaagg atcttcaact agatcctttt aaattaaaaa 5520
tgaagtttta aatcaatcta aagtatatat tagtataact tgtctatttc ggtctgacag ttaccaatgc 5580
ttaatcagtg ctagccctat ctacagcatc tgcctatttc tgtctccat aggttgcctga 5640
ctccccctgc tbtgataaac tacgatcgg gagggtttac catctggccc cagtgtctga 5700
atgataccgc gagaccacg ctacaccggt ccagatttat cagcaataaa ccagccagcc 5760
gggaaggccg agcgagaaag ttggtcctgca actttatccg cctccatcaa gtctattaat 5820
gtttgcgggg aagctagagt aagtagttcg ccagtttaata gttttgcgca cgttgttgc 5880
attgtctacg caactggtgt gtccagctgc tctgtttgta tggctctcat cagctccgg 5940
tcccaacgat caagcgaggt tacatgatcc cccatgttgt tggtccgca tggtatcatt 6000
ttcggctctc cgactggtg cagaagtaag ttgtcccgca tgtttatcag catgtcttct 6060
gcagcactg ataatctct tactgtcatg ccactcgtaa tgcactcttc ttgtactgg 6120
gagtactcaa ccaagtcatt ctgagaatag tgtatgcggc cagcagtgct ctcttgcgc 6180
cggtcaaatc gggataatct cgcgccacat cgcgcacatc taagaagtgt catcatctga 6240
aaacgtcttc cggggcgaaa actctcaagg atctcttca tcttgtagct cagtctgatg 6300
taaccacact gtgcaccaa ctgatcttca gaaatgttta ctctcacag cgtttctggg 6360
tgagcaaaaa caggaaggca aaatgcgcga taattgagga atcagggcag acggaagtgt 6420
tgaatactca tactctcttc ttttcaatat ttatgaggg ttatgtctc 6480
atgagcgagt acatatttga atgtatttg aaaaataaac aaatagggg ttccgcgcac 6540
tttcccgaa aagtgccacc tgacgtc 6567

```

- <210> 12
- <211> 6639
- <212> DNA
- <213> Artificial Sequence

<220>

- <220>
- <223> Description of Artificial Sequence: Construct E2CLDBS

<400> 12

gacggatcgg	gagatctccc	gatccccc	ggtcgactct	cagtaacaatc	tgtctctgatg	60
ccgcatagtt	aagccagtat	ctgctccctg	ctgtgtgtgt	ggaggtcgct	gagttagtgcg	120
cgagcaaaat	ttaagctaca	acaaggcaag	ctgtgacgga	caatgtcgctg	agaactctcg	180
ttaggggttag	gcgcttttgcg	ctgcttcgcg	atgtacgggg	cagatatcacg	cgttgcacatt	240
gtatttgtac	tagttatttaa	tagtaatcaa	ttacggggctc	attagttcat	agcccatata	300
tcggagttcgg	cgcttacaata	cttacggtaa	atggcccgcgc	tggctgcacgcg	cccaacgacc	360
ccgcgccatt	gagctcaata	atgacgtatg	ttcccatagt	aacgcccaata	ggcaactcttc	420
attgacgtca	atgggtggag	tatttaccgt	aaactgcgcc	cttggcagta	catcaagtgt	480
atcatatgcc	aagtagccgc	ccctatgacg	ctaatgacgc	taaatggccc	gctctggcatt	540
atgcccagta	catgacctta	tgggactttc	ctacttggca	gtacatctac	gtattagtca	600
tcgctattac	catgggttga	cggttttggc	agtacaatcaa	tggggctggga	tagccggttg	660
actcacggctc	atttccaagt	ctccacccca	tgcagctcaa	tggggagtctg	ttttggcacc	720
aaaatcaacg	ggactttcca	aaatgtcgta	acaaactccg	cccattgacg	ccaatggccg	780
gtaggcgtgt	acggttggag	gtctataata	ccagagctct	ctgggttaact	agagacacca	840
ctgcttactg	gcttatcgaa	atataacaga	ctcactatag	ggagaccacca	gctggctacg	900
gtttaaactt	aaagcttagat	ctatatggcca	ggcggccctc	gagcccgggg	agaagcccta	960
ctgtgttcgg	gaatgtcgta	agtcctcttc	ctcagagctct	cacctggtgc	gcaccagcgc	1020
taccacacgc	ggtgcaaaac	cgataaaagt	cccagagctg	ggcaaatctc	ttagtgaactg	1080
ccgcgcactc	ctgtcgccatc	aacgcactca	tactggcgag	aagccatata	aaagtccaga	1140
atgtggcgaag	ttcttaccgc	gctctgacaa	ctcgtgtcgt	taccaacgta	ctcacacgta	1200
taaaaaaact	agtgaccgaa	gaggaggagg	aatgttgaaa	cacaagcgta	agagagatga	1260
tgggaggggc	aggggtgaa	tggggtctgc	agagacatg	agagctggca	acctttggcc	1320
aagcccgctc	atgatctaac	gctctaagaa	gaacagcctg	gctctgtcc	tgacggccga	1380
ccagatggtc	agtgccctgt	tggagtgtga	ggcccccata	cttttgtccg	agtatgatcc	1440
taccagaccc	ttcagttgaag	cttcgatgat	gggtctactg	accaacctgc	cagacagggga	1500
ctgggttcaac	atgatctaac	ggcggaagag	ggcgccaggc	tttgtggatt	tgacctcca	1560
tgatcaggtc	ccactttctag	aatgtgcctg	gctagagatc	ctgatagttg	gtctctgtctg	1620
gcgctccatg	gagcacccag	ggaaagctact	gtttgtctct	aaactgtcct	tggaacaggaa	1680
ccagggcaaaa	tgtgtagagat	gcatgggtgga	gatcttcgac	atgtctgtctg	ctacatctatc	1740
tcgggttcgcg	atgatgtatac	tgcacgggaa	ggagtttgtg	tgctctcaat	ctattatttt	1800
ctttaatctc	ggagtgtcga	catttctgtc	cagcacccctg	aagtctctgg	aagagaaggga	1860
ccatattccac	cgagttctgg	acaagatcac	agacactttg	atccacctga	tggcccaaggc	1920
aggccctgacc	ctgcagcagc	agcaccagcg	gctggcccag	ctctctctca	tcctctccca	1980
catcaggcac	atgagttaaca	aaggcatgga	gcatctgtac	agcatgaagt	cgaaagaagt	2040
ggcgcccttc	tatgacatgc	tgctggagat	gctggacgac	cagcccgctac	atgcgccacc	2100
tagccctacg	cccgcccgac	ccctggagca	cttcgacctg	gacatctgac	cgcccgacgcg	2160
cctggagcac	ctgcagactg	acatgctgcc	ggccgacgac	ctggagcagt	tgcagactgga	2220
catgctgcgc	gggttaactaa	gtaagcggcg	gctcgagctc	agagggcccg	tttaaaccgcg	2280
ctgatcagcc	tcgactgtgc	cttctagtgt	ccagccatct	gttgttttgc	ctcccccctg	2340
gccttctctg	acccttggaa	gtgcacactc	caactgcctt	tcctaaataa	atgaggaaat	2400
tgcattcgat	gatttggagta	gggtgtcatt	tattctgggg	gggtgggtgg	ggcaggagac	2460
caagggggag	gatttggagta	acaatagcac	gcatctggg	gctgcgggtg	ctctattggc	2520
ttctgaggcg	gaaagaaacca	gctggggctc	tagggggtat	ccccacgcgc	ccgtgacggg	2580
cgcatataagc	gcgggggggtg	tggttggttac	gcgcagcgtg	accgctaacac	tgcacagcgc	2640
ctcagcgccc	ctcactcttg	ctttcttccc	ttcctttctc	gccacgtctg	ccgggtttcc	2700
ccgtcaagct	ctaaactctg	gcactccctt	aggggttcga	tttagtgctt	tacggcacct	2760
cgcacccaaa	aaacttgatt	aggggtgatg	ttcacgtagt	gggccaatct	ctgtatagac	2820
ggtttttgcg	ccctttgagc	tggaagtccac	gtttcttaat	agtgctacgc	ttgtccaaac	2880
gtgaaacaaca	ctcaaccta	ctctggtcta	ttcttttgat	ttatacggga	ttttggggat	2940
ttcggcctat	gtggttaaaa	ctgagctgat	tttaacaaaa	tttaacggga	atctattctg	3000
tggaaattgtg	gtcagtttag	gtgtggaaag	ctcgcaggct	ccccaggcag	gcacaagtat	3060
cgaaagactg	catctcaatt	agtcagcaac	caggtgtggga	aagtccccag	gctcccacgc	3120
aggcagaagt	atgcaaaagg	tgcattctcaa	ttagtcagca	accatagctc	cgccctaac	3180
tcgcgccatc	ccgcgccctaa	ctccgccagc	ttccgcccat	tctccgcccc	atggctgtgt	3240
aatttttttt	attttatcgag	agggccaggc	cgctctgccc	cgctctgccc	tcacagaagta	3300
gtgagggggc	tttttttggag	gcttaggctt	ttgcaaaaag	ctcccgggag	cttgtatattc	3360
gttttttggga	cttgatcaag	agacaggtat	aggatcgttt	cgcatgattg	acaagaatgg	3420
atttgacagca	gggtctccgcg	ccgcttgggt	ggagaggcta	ttcgggtatg	actgggcaca	3480
acagacaatc	gggtgctctg	atgcccgctg	cttcggcgtg	tcagcgcatg	ggcccgccgt	3540
ctcttttttgc	aagaccagca	gtgcccgtgc	cctgaatgaa	ctgcaggcag	aggcagccgcg	3600
gtctatctgg	ctggcccacc	cgggcgcttc	tttcgacagt	gtgctcgacg	ttgtcactga	3660
agccgggaag	gactgggtcg	tattggcgga	agtgccgggg	caggatctcc	tgctactctca	3720
cctgtctcct	gccgagaaaag	tatccatcat	gtgctgatga	atgcggcggc	tgcatcagct	3780
tgatccgctg	acctgcccat	tcgaccacca	agcgaaatcat	cgcactcgagc	gagcagctat	3840
tcggatggaa	gccgctctctg	tcgatcagga	tgatctggac	gaagagcatc	agggcctcgc	3900
gccagccgaa	ctgttcgcgca	ggctcaaggc	cgcatcgccc	gacggcgagg	atctcgtcgt	3960
gacctatggc	gatgtctcct	tgcccgaatc	tgggtggaa	aatggccgct	ttctgggatt	4020

```

catogactgt  ggcgcgcttg  gtgtggcgga  cgcctatcag  gacatagcgt  tggctaccgg  4080
tgcattgtct  gaagagcttg  ggcggcgaat  ggctgacgcg  ttctctcggt  tttcagggat  4140
cgccgctccc  gatctgcagc  gcatcgccct  ctatcgccct  cttgacgagt  tctcttcgagc  4200
gggactcttg  ggttcgaagt  gaccgaccaa  gcgacgcgca  acctgccatc  ccgagatttc  4260
gattccacgc  cgcgcttcta  tgaaagggtg  ggcttcggaa  togttttcgg  agcgcggcg  4320
tggatgatcc  tccagcgcgg  ggaatcctat  ctggagttct  togcgccacc  caacttggtt  4380
attgcagctt  ataatgggta  ttgtggtttg  tccaaatcga  caaatttcac  aaataaagca  4440
tttttttcaa  tgcatcttag  cttaggcttg  tccaaatcga  tcaatgatct  ttatcatgtc  4500
ttgtatccgt  cgacctctag  cttaggcttg  ggcataatca  ggcataatca  gttctcgtgt  4560
tgaattatct  atccgctcac  aattccacac  aacatacag  ccggaagcag  aaagtgtaaa  4620
gtctgggttg  cctaalgagt  gagctaactc  acattaattg  cgttgcgctc  actgcccgtc  4680
ttccagtctg  gaaaacctgc  gtgcgcagct  cattaatgaa  cgggccaaag  cggcgggaga  4740
ggcggtcttg  ggcgagcgtt  atcagctcac  atcagctcac  tcaaaaggcg  taatacgggt  4800
ttaggggata  acgcaggaaa  gaacatgtga  gcaaaaggcg  agcmetaagg  caggaaaccgt  4860
aaaaaggcgg  cgttctggc  gtttttccat  aggcctcgcc  cccctgacga  gcatcaaaaa  4920
aatcagcgtg  caagtctgag  gtgtcggaac  gctccgaccc  cccctgacga  ccaggcggtt  4980
ccccctggaa  cgttccgtg  gcgtctctct  gctccgaccc  gctccgaccc  cggatcatctc  5040
tcgcgcttcc  tcccttcggg  aagcgtggcg  ctcttctca  gctccgaccc  taggtatctc  5100
agttcggttg  aggtcggttc  ctccaagctg  ggtgtgtgtc  acgaaccccc  cgttcgcccc  5160
gacgcgtcgc  ccttatccgg  taaactatcg  cttagtcca  acggcgtaag  acagactta  5220
tcgccacttg  cagcagccac  ttgtaaacag  attagcagag  cgaggtgatg  agcgggtgct  5280
acagagtctt  tgaagtgggt  gctcaactac  ggctacacta  gaaggacagt  atttggatc  5340
tgccgtctgc  tgcagcagct  taccttcgga  aaaagagttg  tgactctttg  atccggcaaa  5400
caaacacccg  ctggttagcg  tgggtttttt  gtttgacaga  agcagacaga  ggcgcgaaaa  5460
aaaggatctc  aagaagatcc  tttgatcttt  tctacggggt  ctgacgcttc  gtggaaacgaa  5520
aactacagtt  aagggtattt  ggtcatgaga  ttatcaaaaa  agatcttcaa  ctatagcttt  5580
ttaaattaaa  aatgaagttt  taaatcaatc  taaagtata  atgagttaac  ttggtctgac  5640
agttaccaat  gcttaatcag  tgaggcacct  attcagcaga  tctgtctatt  tctgtctacc  5700
atagttgctc  gactccccgt  cgtgtagata  actacgatac  gggagggggt  accatctggc  5760
ccagtgctg  caatgatacc  gcgagaccca  cgctcacggg  ctccagattt  atcagaata  5820
aacccagcag  ccggaaggcg  cgagcgtaga  agtggctctg  caactcttat  cgctccatc  5880
cagctctatta  attgttcggc  ggaagctaga  gtaagtattg  cgccagttaa  tagttttggc  5940
aacgttgttg  ccatctgtac  aggcacgtg  gtgtcacgct  cgtcgttttg  tatggcttca  6000
ttcagctcgc  gtttccaaag  atcaaggcga  gttacatgat  cccccatgt  tatggcctaa  6060
cgcggttagct  ccttcggtcc  tccgatcggt  gtcagaagta  agtttgccgc  agtgttatca  6120
tcaggtgtta  tggcagcact  gcataattct  ctactgtca  tgccatccgt  aagatgcttt  6180
tctgtgactg  gtgagtactc  aaccaagtca  ttctgagaat  agtgatagc  ggcagccagt  6240
tgctcttgcc  cggcgctca  acgggataat  acggcgccac  atagcagaag  tttaaaagtg  6300
ctcatctatt  gaaaaacctc  tctgtgaccc  aaactctcaa  ggatcttacc  gctgttgaga  6360
tcaggttcga  tghtaacccac  tctgtcaccc  aactgatctt  cagatcttcc  tctcttcaac  6420
agcgtttctg  ggtgagcaaa  aacaggaagg  caaaatgccg  caaaaaagg  aataaggggc  6480
acacggaaat  gttgaatact  catactcttc  ctttttcaat  attattgaag  catcttatcag  6540
ggttattgtc  tcatgacggc  atacatattt  gaattgtatt  agaaaaataa  acaaataggg  6600
gttcgcgcga  tcttcccccg  aaaagtgcga  cgtgacgtc  gctgacgtc  gttattgca  6639

```

&lt;210&gt; 13

&lt;211&gt; 6801

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;220&gt;

<223> Description of Artificial Sequence: Construct  
LBDASNLVSP16

&lt;400&gt; 13

```

gacggatagg  gagatctccc  gatccccat  ggtcgactct  cagtacaact  tgcctgtgat  60
cgcgatagtg  aagccagtat  ctgctccct  ggtgtgtgtt  ggaggtcgct  gtagtagctg  62
tcaggcaaaa  ttaagctaca  acaaggcaag  gcttgacgga  caattgcagt  cgaatactgc  64
cgaggtttag  cgcgttttgc  ctgcttcgg  atgtacgggc  cagatatacg  agtgaattct  66
gattattgac  tagttattaa  tagtaatcaa  ttacgggttc  attagttaac  agccatata  68
tggagtctcc  cgtaacataa  ctacggtaa  atggcccgcc  tggctgcagc  ccaacgacc  300
ccgcgccatt  gacgtcaata  atgacgtat  ttccatagt  aacgcaata  gggactttcc  420
attgacgtca  atgggtggac  tatttacgtg  aaactgccca  cttggcagta  catcaagtg  480
atcatatgcc  aagtacgccc  cctattgac  tcaatgacgg  taaatggccc  gctcggcatt  540
atgcccagta  catgacctta  tgggacttcc  ctacttgga  gtacatctac  gttattgca  600

```

tcgctattac	catggtgatg	cggttttggc	agtacatcaa	tgggcgtgga	tagcggtttg	660
actcacgggg	atttccaagt	ctccaccccc	ttagcgtcaa	tggggagtgtg	ttttggcagg	720
aaaatcaacg	ggactttcca	aaatgctgta	acaactccgc	cccatgtacg	caattggcgc	780
gtaggcgtgt	acgggtgggg	gtctatataa	gcagagctct	ctggcttaact	agagacacca	840
ctgcttaagt	gctttacgaa	atataacgaa	ctcactatag	ggagaccgca	gcgggtcagc	900
gtttaaactt	aagctttgta	ccgagctcgg	atccactagt	ccagttgtgtg	ggaaatctctg	960
cagcccgggg	gatctatggc	ccaggcgggc	ctcgagccct	atctgtggcc	tgtcgagattc	1020
tgcgatgcgc	gctttttcaa	gtcggctgat	ctgaagcgcc	atataccgat	ccacacaggc	1080
cagaagcctt	tcaggtttcg	aaatagcatg	ctgaacttca	gtcgtagtgt	accacttaac	1140
accacacatc	gcaccacacg	aggcgagaag	ccttttgctc	gtgacatttg	tggggaggaag	1200
tttgcgcagg	gtgatgaaag	caagaggcat	accaaatacc	atttaagaca	gagggcatct	1260
agaactagtt	ctcgttggaa	catgagagct	gccaaacttt	ggccacagccc	ggtcatgatc	1320
aaacgctcta	agaagaacag	ctcggccttg	ctcgtacagg	ccagccagatg	gttcagtgccc	1380
ttgttggtat	ctgagccccc	catcacttat	tccgagtatg	atccctacat	acccttcagt	1440
gaagcttcga	tgtatggctg	actgaccacg	ctggagagca	gggagctggg	tcacttcagt	1500
aactggggcga	agcgggtggc	aggttttggt	gatcttgacc	ctcatgatca	ggttcaacct	1560
ctagagctga	octggctaga	gactcctgat	atgtgtctcg	tctggcgctc	catggagacc	1620
ccagggaagc	taactgtttg	tcctaacttg	ctcttggaca	ggaacacagg	aaaatgtgta	1680
gaggcgcatg	tggagatctt	cgacaatgct	ctggctacat	catctcggtt	ccgcattgatg	1740
aatctcgacg	gagaggagtt	tgtgtgcctc	aaatctatta	ttttgcttaa	tcttgaggtg	1800
tacacatttc	tgtccagcac	octgaaagct	ctggaagaga	aggacacata	ccaccgagtc	1860
ctgcacaaga	tcacagacac	tttgatccac	ctgatggcca	aggcaggctg	gaccttcgac	1920
cacgacgacc	agcggcttgc	ccagctcctc	ctcatcctct	ccacatgcct	gcacatgagt	1980
aaacaaggca	tgggcatctg	gtacagcatg	aagtgcgaaga	acgtgtgtgc	ctcttatgac	2040
ctgctgctgt	agatgcttga	gcgccaccgc	ctcacatgcg	ccactacggc	tcaggccgaa	2100
aagaaaacga	aagtgtggcg	cgccggcgct	cccccgacgc	atgtcagcct	gggggtcagag	2160
ctccacttag	acggcgagga	cgctggcctg	gcgcgatccg	acgcgcctaga	cgatttcgat	2220
ctggacatgt	tggggggacg	ggatttcccc	ctccggggat	ttaccctcca	gcactccgcc	2280
ccctacggcg	ctctggctat	ggccgacttc	gagtttgagc	agatgcttaa	cgatccctct	2340
ggaatttgac	agtaacggtt	aattaactac	ctccagcagc	tctccgacta	cgcttcttga	2400
gaatttcggg	cgcttcgagt	ctagagggac	cgtttaaacc	cgctgatcac	ctctgactgt	2460
tatctctagt	tggccacgat	ctgtttgttt	cttccctccc	gtctctctct	tgcacctgga	2520
aggtgccaat	cccaactgtc	tttcttaata	aaatgaggaa	attgcatcgc	atttgtctga	2580
taggtgtcat	tctatctctg	ggggctgggt	ggggcaggac	ttacgaaggg	aggatttggga	2640
agacaaatgc	aggcatcgtg	gggatgcggt	gggctctatg	gctcttgagg	cggaaaagac	2700
cagctggggc	tctagggggg	atccccacgc	gcccgttagc	ggccgattaa	cggtcgccgg	2760
tgtggttggt	acgcgcagcg	tgaccgctac	acttgccagc	gcccctagcg	ccgctctctt	2820
cgctttcttc	ccctcccttc	tgcgacagtt	cgccggcttt	cccctgcaag	ctctaaatcg	2880
ggggcatcct	ttagggtttc	gatttagtgc	tttacggcac	ctcgacccca	aaaaacttga	2940
ttaggttgat	gggttcagta	gtggggcctc	gcccgtgatg	acgggttttc	gccccttgac	3000
gttgagttcc	acgttcttta	atagtggact	ctgttcccaa	actggaaaca	cactcaaccg	3060
tatctcgctc	tattcttttg	atttataagg	gattttgggg	atctcgccct	attgtttaaa	3120
aaatgagctg	attttaacaa	aatttaacgc	gaattaaatc	tgtggaatgt	gtgtcagtta	3180
gggtgtggaa	gtgtccacag	ctccccagcg	aggcagaagt	atgcaaaagca	tgcattccaa	3240
ttagtacaga	accagggtgt	gaaagtcccc	aggctcccca	gcaggcagaa	gtatgcagaa	3300
catgcatctc	aattagtcat	caaccatagt	ccgcgcccta	actccgccca	tcocgcgcct	3360
aaactccgcc	agtttccgcg	atcttccgcg	ccatggctga	ctaatatttt	ttatttatgc	3420
agaggcgag	gcgccctctg	actctgagct	atccagaag	tagtgaggag	gtcttttttg	3480
aggctcagg	tttttcaaaa	agctcccggt	agctgtata	tccactctcg	gatctgatca	3540
agagacagga	tgaagtacgt	ttcgcactgat	tgaacagagt	ggatttgacg	caagttctcc	3600
ggcgcgttgg	gttgaaagcg	tattcggcta	tgaactggga	caacagacga	ctggctgctc	3660
tgatgcgcgc	gtgttccgcg	tgtcagcgca	ggggcgccgc	gttcttttgg	tcaagacgca	3720
ctctgtcctc	gccttcaaat	aactgcagga	agggcagcg	ggctactcgt	gtgtggccac	3780
gacggggcgt	ccttgcgcag	ctgtgctcga	cgttgtcac	gaagcgggaa	gggactggct	3840
gctatttggt	gaaagtgcgg	ggcagatct	ctctgcatc	cacctctgtc	ctcccgagaa	3900
agttatccat	atgggtgatg	caatgcggcg	gctgcatacg	cttgatccgg	ctaccctgcc	3960
atttcgacac	caagcgaaat	atcgatcga	gcgagcacgt	actcgatgat	agacccgtct	4020
tgtcgatcac	gatgatctgg	acgaagagca	tcaggggctc	gcgcacagcg	aactgttcgc	4080
caaggctcaag	gcgcgcatcg	ccgacgcgca	ggatctctgc	gtgacccatg	gcagtcctg	4140
cttgccgaagt	atcatgttgt	aaaaatggcg	cttttctgac	ttcatcgact	gtggcgcgct	4200
gggtgtggct	gaccgctatc	aggacatagc	gttggtctacc	cgtagacttg	ctgaagagct	4260
tggcggcgaa	tgggtatcgc	gcttccctcg	gctttacggt	atcgcgcgct	cggtatccga	4320
gcgcatcgcc	ttctatcgcc	ttctttgacg	gttcttctga	cggggactct	gggttgcgaa	4380
atgaccgcgc	aagcgacgcc	caacctgcc	tcacgagatt	tcatattccac	cgccgccttc	4440
tatgaagact	tgggcttctg	aatcggtttc	cgggacgcgc	gctggatgat	ctcccaagcg	4500
ggggatctca	tgttggagtt	cttcgcccac	cccaacttgt	ttaattgcagc	ttataatggt	4560
tacaaataaa	gcaatagcat	acaaaatttc	acaaaataag	ttattttttc	atcgtactct	4620

```

agttgtgggt  ttgtccaaact  catcaatgta  tcttatcatg  tctgtatacc  gtgcagcctc  4680
agctataggt  ttggcgtaact  atgggtcatg  ctgttttctg  tgtgaaattg  ttatccgcctc  4740
acaattccac  acaacatcac  agccgggaag  ataaagtgtg  aagcctgggg  tgacctatga  4800
gtgagctaac  tcacattaat  tgcgttgcgc  tcaactgcgc  ctttccagtc  gggaaacctg  4860
tctgtccagc  tgcattaatg  aatcgcccaa  cgcgcgggga  gaggcgggtt  gggcgagcgc  4920
gtatcagctc  actccaaaagg  ggtaatacgg  ttatccacag  aatcaggggg  taacgcagga  4980
aagaacatgt  gagcaaaaagg  ccagcaaaaag  gccaggaaac  gtaaaaaggc  ccgcttgctg  5040
gcgtttttcc  ataggctccg  cccccctgac  gagcatcaca  aaaaatcgag  ctcaagtcaag  5100
agggtgggaa  acccgacagg  actataaaga  taccaggcgt  ttccccctgg  aagctccctc  5160
gtgcgtcttc  ctgttcggac  cctgcgcctt  accggatacc  tgctccgctt  tctcccttcg  5220
ggaaagctgg  cgtcttccca  atgtctcacg  tgtaggatct  tcaagtccgt  ctaggtcgtt  5280
gcctccaaag  tgggtgtgtg  caaccgggta  ccggttcagc  ccgaccgctg  cgccttatcc  5340
ggtacatctc  actcgttaaca  ggaattgacg  agcagggtat  tagaaggaca  ggcagcagcc  5400
tggtccaaact  accgctcacac  agcgttcacg  gatctgtcta  ttaccatctg  cttgaaagtg  5460
gttacctctg  gaaaaaagag  tggtagctct  tgcgtcgtct  tctgcgctct  gctgaagcca  5520
gggtgttttt  ttgtttcgaa  gcagcagatt  acgcgcggga  aaaaaccacg  cctggtagcg  5580
cctttgatct  ttctatcggg  gtctgacgct  cagtggaacg  aaaaactcag  ttgaaggagt  5640
ttggtcatga  gattatcaaa  aaggatcttc  acctagatcc  ttttaaatat  aaaaatgaag  5700
tttaaatcaa  ttaaaagtat  atatgagtaa  acctgtgtct  acagttagct  atgcttaact  5760
agtgaggcac  ctatctcagc  gatctgtcta  ttctgtctat  ccaatagtgc  ctgaactccc  5820
gtctgtgaga  taactacgat  accggagggc  ttaccatctg  cccccagtcg  tgaagtgctg  5880
cccgagagac  cagcgtccac  ggctccagat  ttatcagcaa  taaaccagcc  agccggaagc  5940
gccagcgcaa  gaagtgtctc  tgcaacttta  tccgcctcca  ccaactgttg  tgcaattgtc  6000
cgggaaagcta  gagtaagtat  ttgcacagtt  aatagtttgc  gcaacgttgt  tgcatttctc  6060
acaggcatcg  tgggttcacg  ctctgtcttt  ggtagtgctt  tctcagctct  cggttcccaa  6120
cgatcaaggc  gaggtaacat  atccccatct  ttgtgcaaaa  aagcgttgtt  ctctctcgtg  6180
ctcccgatcg  ttgtcagaag  taagtgtggc  gcaagtgtat  caactatggt  ttgtgtgacg  6240
ctgcataatt  ctcttactgt  catgccatcc  gtaagtgtct  tttctgtgac  ttgtgtgacg  6300
tcaacaacgt  cattctagga  atagtgtatg  cggcgaccga  gttctctctg  cccggcgctc  6360
atacgggata  ataccgcggc  acatagcaga  actttaaaag  tgctcatcat  tggaaaacgt  6420
tctctggggc  gaaaaactct  aaggatcttc  cgcgtgttga  gatcagagtc  gatgttaacc  6480
actcgtgcac  ccaactgatc  ttacagcatc  tttactttca  ccagcgtttc  tgggtgagca  6540
aaaaacggaa  ggcaaaaatg  cgcacacaaa  ggaataaagg  cgacacggaa  atgttgaata  6600
ctcatactct  tctcttttca  atattattga  agcatttato  aggggtattg  tctcatgagc  6660
ggatacatat  ttgaattgat  ttagaaaaat  aacacaaatg  ggggtccgcg  cacatttccc  6720
cgaaaagtgc  caccctgagc  c  6780

```

```

<210> 14
<211> 6695
<212> DNA
<213> Artificial Sequence

<220>

<220>
<223> Description of Artificial Sequence: Construct
LBD5G400V

```

```

<400> 14
gacggatcgg  gagatctccc  gatccccat  ggtcgactct  cagtacaatc  tgctctgatg  60
ccgcatagtt  aagccagtat  ctgctccct  ctgtgtgtgt  ggaggtcgct  gagtatgctg  120
cgagcaaaat  ttaagctaca  atcgcccaa  gcttgaccga  caattgcacg  aagatctcgt  180
ttagggttag  cggtttttgc  ctgcttcgc  atgtacgggc  cagatatacg  cgttgacact  240
gattattgac  tagttattaa  tagtaataca  ttacggggtc  attagtctat  agccatata  300
tggagtctcc  cgttacataa  cttaacggta  atggcccgcc  tggctgaccg  cccaacgacc  360
cccgccactt  gacgtcaata  atgacgtatg  ttcccatagt  aacgccaata  gggactttcc  420
attgacgtca  atgggtggac  tatttaacgt  aaactgcoca  ctggcgagta  catcaagtgt  480
atcatatgcc  aagtacgccc  cctattgacg  tcaattgaag  taattggccc  ccaatggctt  540
atgccacgta  catgaacctta  tgggactttc  ctacttgcca  gtacatctac  gtattagtca  600
tcgctattac  catggtgatg  cggtttttgc  agtacctcaa  tgggctggga  tagcgtttcc  660
actcaccggg  atttcccaag  ctccacccca  ttgaagtcac  tgggagtttg  ttttggcacc  720
aaaaatcaag  ggaatttcca  aaatgtcgta  atcaactccg  cccattctgc  caaatgggag  780
gtaggcgtgt  acggtgggag  gtctatataa  gcagagctct  ctggctaaat  agagaaacca  840
ctgcttaact  gcttatcgaa  attaatacga  ctcaactatg  ggagacacaa  gctggtcagc  900
gttttaacct  aagcttggtg  ccagagctgc  atccactagt  ccagtggtgt  ggaattctcg  960
cagcccgggg  gatctatggc  ccaggcgccc  ctcgagccct  atgttgcgcc  tgtcgagctc  1020

```

tgcgatcgcc	gcttttctaa	gtcggctgat	ctgaagcgcc	atatccgcat	ccacacagcg	1080
cagaagcctt	tccagctgtc	aatatgcgat	cgtaacctta	gtcgtagtag	ccaccttacc	1140
accoccatcc	gcacccacac	agggcgagaag	ctcttttgctt	gtgacatttg	gtggaggaaag	1200
ttgtgccagga	gtgatgaaacg	caagaggcat	acccaaaatcc	atttaagaca	gagggactct	1260
agaaacttga	accgaagagg	agggagaagt	ttgaaacaca	agcgccagag	agatgatggg	1320
gagggcgagg	gtgaagtggg	gtctgtcgga	gacatgagag	gtgcaccaact	gtggcccaagc	1380
ccgctcatga	tcaaacgctc	taagaagaac	agcctggcct	tgctccctgac	gtccgcacag	1440
atggctcag	cctttgttga	tgctgagccc	cccataactct	atcccgagta	tgatcctacc	1500
agacccttca	gtgaagctcg	gatgtggggc	ttaactgacca	actcggcaga	cagggagctg	1560
gtccacatga	tcaactgggc	gaagaggggt	ccaggcttttg	tggatttgac	ctctcatgat	1620
caggtccacc	ttctagaatt	tgctctgcta	gagatcctga	tgattgtgtg	cgctctggcg	1680
tccatggagc	accocagtaa	gctactgttt	gctccttaact	gtctctgtga	cgagcaacag	1740
ggaaaatttg	tagagggcat	ggtgtgagat	ttcgacatgc	tgctgtgctac	atcatctcgg	1800
ttcccgatga	tgaattctga	gggagaggag	tttgtgtgcc	tcaaatctgt	tatttttgct	1860
aatcttcgag	tgtaacacat	ttctgtccag	accctgaagt	ctctggaaga	gaaggacatc	1920
atccaccgat	tcttcggaca	gatcacagac	actttgatcc	actctgattc	caaggcaggc	1980
gtgaccctgc	agcgacagca	ccagcggctc	gccacgctcc	ttctcatcct	ctcccaactc	2040
aggcacatga	gtaaacaaag	catgtgagcat	ctgtacagca	tgaagtgtcaa	gaacgttggt	2100
ccctctcgag	acctgtctct	ggagatgctg	gacgcccacc	gcctacatgt	gcccatcagc	2160
cgtacgcggg	ccgacgctct	ggacgacttc	gactgtgaca	tgcgtccggc	cgacgcctgt	2220
gcgcagctct	acctggacat	gctgcgggct	gacgcccctg	acgacttcga	actggacatg	2280
cagccggggg	aactaagtaa	gcggccgctc	gagctatagag	ggcccgctta	ccctcgctga	2340
tccgcttcga	ctgtgccttc	tagttgccag	ccatctgttg	tttgcctctc	cccctgtcct	2400
tcacttgacc	tggaagtgtg	cactcccatc	gtcctttctc	aataaataga	ggaaatttga	2460
tgcattgttc	tgagttaggt	tcattctatt	ctgggggggt	gggtggggga	ggacacgaag	2520
ggggagagtg	gggaagacaa	tagcaggcat	ctgtggggat	ccgttgggctc	tatgtctctc	2580
gggacggaaa	gaaccagctg	gggctctagg	gggtatcccc	acgcgccctg	tacgcggcca	2640
ttagcgcggg	cggtgtgtgt	ggttacgcgc	agcgtgaacc	ctacacttgc	gagccctcta	2700
gcgcgcgctc	cttttcgctt	cttcccttcc	tttctgccta	cggtgcggcg	ctttcccgct	2760
caagctctaa	atcggggcat	ccctttaggt	ttccgattta	gtgcttttac	gcactccgac	2820
cccaaaaaaa	ttgattaggg	tgatggttca	cgtagtgggc	catcgccctg	atagactgtt	2880
tttgcctctt	tgacgtttga	gtccacgttc	tttaaatagt	gactcttgtt	ccaaactgga	2940
acaaacactca	accctatctc	ggtctattct	tttgatttat	aagggtatct	ggggatttgc	3000
gcctatttgg	taaaaaatga	gctgatttaa	caaaaattta	acgcaattta	atgtcttgga	3060
atgtgtgtca	gtttaggggt	ggaaaagccc	caggctcccc	agggcaggca	aagtatgcaa	3120
agatgatctc	tcaatttagtc	agcaaacagc	tgtggaaaag	ccccaggctc	ccagcaggcg	3180
agaagtatgc	aaagcatgca	tctcaattag	tcagcaacca	tagtcccgcc	ctcaactccg	3240
cccatccgcg	ccctaactcc	gccacgttcc	gcccatctct	cgccccatgt	ctgaactaat	3300
ttttttattt	tcgacagagg	cgaggccgcc	tctgcctctg	agctatttca	gaagtatgta	3360
ggagctcttt	ttggaggcct	aggcttttgc	aaaaagctcc	cgggagcttg	tatatccatt	3420
ttcggatatt	atcacaagag	aggatgagga	tgcttttcga	tgattgaaca	agatgatttg	3480
ccgcagagtt	ctccggcgcg	ttgggtggag	aggctattcg	gctatgactg	ggcacacaag	3540
acaatcgagt	gctctgatgc	cgccgtgttc	cggctgtcag	cgcaggggcg	cccgtttctt	3600
tttgtcaaga	cggtgccttc	cggtgccttc	aatgaactgc	aggacgagcg	agcgcgctca	3660
tcgtggctgg	ccagcagcgg	cgttccttgc	gcagctgtgc	tcgacctgtg	cactgaagcg	3720
ggaaaggact	ggctcgtatt	ggggcgaagt	cgccggcgagg	atctcctgtc	atctcacctt	3780
gctctgcggc	agaaagactg	catcatggct	gatgcaatgc	ggcgcttgca	tacgcttgat	3840
ccggctacct	gccactctga	ccaccaaagc	aaacatcgca	tcagcgagc	acgtactcgg	3900
atgggaacgc	gtcttgtctga	tcaggatgat	ctggagcaga	agcatcaggg	gctcgcgcga	3960
gcgaagaact	tcgccaggct	caaggcgctg	atgcgccgag	gcgaggatct	cgctctgtacc	4020
catcgcgatg	ctcgactgtc	gaatatcatg	gtggaaaatt	tgctctgttg	tgactcgtgat	4080
gactgtggcc	ggctgggtgt	ggcgagccgc	tatcaggaca	tagcgtgttg	tacccgtgat	4140
attgctgaag	agcttgcggg	cgaatgggct	gagcgtctcc	tgctgtttta	ctactgtccc	4200
gtccccgatt	cgacgcgatc	cgccctctct	cgccctcttg	acgagattct	ctgagcggga	4260
ctctgggggt	cgaaaatgac	gaccaagcga	cgcccaacct	gccatcacga	gatttcgatt	4320
ccaccgcgcg	cttctatgaa	aggttgggct	tcggaaatgt	tttcggggag	gcccgtctga	4380
ggagctccca	gcgcggctat	ctcatctgct	agttcttctg	ccaccccaac	tttctttatt	4440
cagcttataaa	tggtttacaaa	taaaagcaata	gcatcacaaa	tttcacaaa	aaagcatctt	4500
ttcaactgca	ttctagtgtg	ggtttgtcca	aatcatcaca	tttctcttat	catgtctgta	4560
tacogtgcag	ctctagtgat	agcttggcgt	aatcatggct	atagctgttt	ctctgtgtaa	4620
attgttatcc	gctcacattg	ccacacaaca	tacgagccgg	aagcataaag	tgttaaagcct	4680
gggggtgcta	atagtagtag	taactcacat	taattgcgtt	gcgctcaact	cccgtcttcc	4740
agtcgggaaa	ccctgtcgtg	cagctgcatt	gaatgaatcg	ccaacgcgcg	gggagagcgc	4800
gttttcggcg	agcggtatca	gctcactcaa	agggcgtaat	acggttatcc	acagaaatga	4860
gggataacgc	agggaaagaac	atgtgagcaa	aaggccagca	aaaggccagg	aacctgtaaa	4920
agggcgcgct	gctggcgttt	ttccatagcg	tcggccccc	tgacagagat	cacaaaaact	4980
gagctcatga	tcagaggtgg	cgaaaaccga	caggactata	aagataacag	gctttcccc	5040



gttcacatga	tcaactgggc	gaagagggtg	ccaggctttg	tggatttgac	cctccatgat	1620
cagggtccac	tcttagaatt	tgccctggcta	gagatcctga	tgatttggtc	cgctcggcgc	1680
tccagtgagg	acctgaaggaa	gctactgttt	gctccttga	tgctcttgga	caggaaaccag	1740
ggaaaaattg	tagaggggcat	gggtggagatc	ttcgacatgc	tgctgggtcac	atcatctcgg	1800
ttcccgatga	tgaattctgca	gggagaggag	tttgtgtgcc	tcaaatctat	tattttgctt	1860
aatctctggag	tgtcacacatt	tctgtccagc	accttgaagt	ctcttggaaga	gaaggaccatt	1920
atccaccgag	tctcgagcaa	gatcacagac	actttgatcc	acctgatggc	caaggcgaggc	1980
gtgacctcgc	agcagcagca	ccagcggctg	gcccgagctc	tcctcatcct	ctccacattc	2040
aggcacatga	gtatacaaac	catggagcat	ctgtacagca	tgaagtgcga	gaacgttggtg	2100
ccctctcatg	acctgtgctc	ggagatgctg	gacgccacc	gcctcatatg	gcccatcagc	2160
ctctacgcgc	ccgacgcctc	ggacgacttc	gacctggaca	tgctgcgcgc	cgacgcctcg	2220
gacgactctg	acctggacat	gctgcggggc	gacgccctgg	acgacttcga	cctggacattg	2280
tgctcggggg	aactaaagtta	goggccgctc	gagtcctagag	ggcccggtta	aaacccgtgta	2340
tcagctcgga	ctgtgccttc	tagttgcagc	ccatctgttg	tttgcctctc	ccccgtgctc	2400
tccttgagcc	tggaagggtg	cactcccatc	gtcctttcct	aataaaatga	ggaaattgca	2460
tcgcatgttc	tgaagtgtg	tcatctcatt	ctgggggggtg	gggtgggggca	ggacagcaag	2520
ggggagattt	gggagagcaa	tagcaggcat	gctgggggatg	cgctggggctc	tatggctctc	2580
gaaggcgaaa	gaacgcgtgc	gggtctcagg	gggtctccgc	acgcgcctcg	tcgcggcgca	2640
ttaaagcggg	cggtgtgtgtg	gggtacggcg	agcgtgacgc	gcacacttgc	gacgcgccta	2700
ggccgcctgc	cttctgcttt	cttcccttcc	ttctccgcca	cttctccggg	cttctccggc	2760
caagctcttaa	atcggggcat	ccctttaagg	ttccgattta	gtgctttacg	gcacctccgc	2820
cccaaaaaaa	ttgattaggg	tgatggttca	cgtagtgggc	ctacgcctcg	atagacgggt	2880
tttcgcctct	tgactgttga	gtccacgttc	tttaaatagt	gactcttgtt	ccaaactgga	2940
acaacactca	acctatcttc	ggctctattt	tttgaattat	aagggaattt	gggaattctcg	3000
gcctatttgtt	taaaaaattga	gctgatttaa	caaaaaattta	acgcgaattta	attctgtgga	3060
atgtgtgtcta	gttaggggtg	ggaaaagtcca	caggtctccc	aggcaggcaga	aagtatgcga	3120
agcatgcatc	ctcaattagt	agcaaacagg	tgtggaaagt	ccccaggctc	ccacgcaggc	3180
agaagtatga	aaagatgcga	tctcaattag	tcgaacaacca	cttcgcgcgc	cttaactcgc	3240
cccatcccg	ccctaactcc	gccacgttcc	gccactctc	cgcccaatct	ctgaactaatt	3300
ttttttattt	atcgagagcc	cgaggccgcg	gctgcctctg	acgtattcca	gaagtattga	3360
ggagctcttt	ttggaagcat	aggcttttgc	aaaaagctcc	cgaggagcttc	tatatccatt	3420
ttcggtattt	atcaagagac	aggatgagga	tctgtttcga	ctatgaaaca	agatgagattg	3480
cacgcaggtt	ctccggcgctc	ttgggtggag	aggctatttcg	gctatgactg	ggcacaacag	3540
acaatcgctc	gctctgcatg	cgccgtgttc	cgctgtccag	cgagggggcg	cccggtttct	3600
tttgtcaaga	ccagacctgtc	cggtgccctg	aatgaactgc	aggaagaggc	agcgcggcta	3660
tcgtggctgg	ccacgagctg	cgttccttgc	cgactgtgc	ctgaagtgt	ctcgaagcg	3720
ggagagtgct	ggctcgtatt	ggggcgaagt	cgggggcagg	atctcctgtc	atctcacctt	3780
catcgctgcg	agaaagatc	catcatggct	gatgcaatgc	ggcggtcgca	tacgttgtat	3840
ccggctacct	gcccaattcga	ccaccaagcg	aaacatcgca	tcgagcgagc	acgtatcgcc	3900
atggaagcgc	gtcttgtcga	tcaggatgat	ctggacgaag	agcatcaggg	ctcgcgcga	3960
gcccgaactg	tcggcaggct	caaggcgccg	atgcccagcg	gcgagatcat	cgctgtgacc	4020
catggcgatg	gtcttgtgcc	gaatatcatg	gtggaaaatg	agcacttttc	tggattcacc	4080
gactgtggcc	ggctgggttg	ggcggacgcg	tatcaggaca	tagcgtttgc	taccctgat	4140
attgctgaag	cgaaatggcg	cgaaatggct	cgccgtcttc	tcgtgcttta	cggtatcgcc	4200
ctctcccgat	cgacgcgatc	cgccctctat	cgctctttg	acgagtttct	ctgagcggga	4260
ctctgggggt	cgaaatgacc	gaccaagcga	cgcccaacct	ggcatacaga	gatttccgat	4320
ccaccgcgcg	ctctctatga	aggttgggct	tcggaatgct	ttcttgggga	gcgcggtgga	4380
tgatctccca	ggcgggggat	ctcatgctgg	agtctcttcg	ccacccccac	ttgtttattg	4440
cagcttatata	tggttacaaa	taaaagcaata	gcatcacaaa	tttcacaaa	aaagcatttt	4500
tttcaatgca	ttctagttgt	ggtttgtcca	aacctacaaa	ttgatcttat	ctctgtctga	4560
taccgtcgac	ctcagatgat	ccacaacaaa	ctacagagct	atagctgttt	ctgtgtgtgaa	4620
attgttatct	gctocaaatt	taactcaaat	taagcaacgc	aagcatctg	tttgaagcct	4680
ggggtgtgcta	atgagttgag	caagctgcat	taattcggtt	ggcgtcactg	cccgctttccg	4740
agtcggggaaa	ctctgtcgtgc	caagctgcat	aatgaatcgg	ccaacgcgcg	gggagagggc	4800
gttttgcgcg	agcggatca	gctcaactaa	agcgggtaat	acggttatcc	acagaatacg	4860
gggataacgc	aggaagaagc	atgtgagcaa	aaggccagca	aaaggccagg	aaccgtaaaa	4920
agcccgctgt	gctggcgttt	ttccataggc	tcggccccc	tgacgagcat	cacaaaagct	4980
gacgtctcaag	tcagaggttg	cgaaaaccga	caggactata	aagataccag	gcgtttcccc	5040
ctggaagctg	ctcgttgctg	tctcctgttc	gcagcctgcc	gtctaccgga	tactcgtccg	5100
ctctttctccc	ttcgggaagc	gtggcgcttt	ctcaatgctc	acgctgtagg	tattctcagt	5160
cggttgtagg	cgcttgctcc	aaagctggct	gtgtgcacga	accccgctg	cagccgcacc	5220
gctgcgcctt	atccggtaac	tatcgtcttg	agtcacaccc	ggtaagacac	gacttatcgc	5280
cacggcagc	agccactga	aacagattta	gcagagcgag	gtatgtagcg	ggtgtcacag	5340
agtcttgtgaa	gtgggtggctt	aactacggct	acactagaag	gacagtattt	gggtatctcg	5400
ctctcgtgaa	gccagtttaac	ttcggaaaaa	gagtttgtag	ctcttgatcc	ggcaaaaaaa	5460
ccaccgctgg	tagcgggtgtg	ttttttgttt	cggaagcgca	gattacgcgc	agaaaaaaag	5520
gatctcaaga	agatcctttg	atcttttcta	cggggtctga	cgctcagttg	aacgaanaact	5580

cacgttaagg	gattttgggtc	atgagattat	caaaaaggat	cttcacactag	atccttttaa	5640
attaaaaatt	aagtttttaaa	tcaattctaaa	gtatatatga	gtaaaacttgg	ctcgacagtt	5700
accaatgctt	aatcagtgag	gcacactatct	cagcgatctg	tcattttcgt	tcattccatag	5760
ttgctcgact	ccccgtcgtg	tagataacta	cgatacggga	gggtcttacc	cttggcccca	5820
gtgctgcgaat	gatacgcgga	gaccacagct	caccgctcct	agattttatca	gcatttaaac	5880
agccagccgg	aagggccgag	cgcagaaagt	gtcctgcacac	tttatcccgcc	tcattccagtt	5940
ctattaaatt	ttgcccgga	gctagagtaa	gtagtctgcc	agtttaaagt	ttggccaacg	6000
ttggttgccat	tgctacaggg	atcgtgggtg	cacgctcgct	gtttggtagt	gcttcatctca	6060
gtcccggttc	ccaacggttc	agggcagttg	catgatcccc	catgtgtgtgc	aaaaaagcgg	6120
ttagctctctt	cggtcctccg	atcgtttgtca	gaagtaagtt	ggccgcagbtg	ttatcactca	6180
ttggttatgg	agcacttgat	aattctctta	ctgtcatgcc	atccgttaaga	gtctttctgt	6240
tgactcgttga	gtactcaacc	aagtcattct	gagaatagtg	tatggcgcgga	ccgagtttgtc	6300
cttgcccgcc	gtcaaatcgg	gataaatccg	gcgccacatag	cagaactctta	aaagtctcca	6360
tcattggaaa	acgttctcttc	ggggcgaatac	tctcaaggat	cttaccgcgtg	ttgagatcca	6420
gttcgatgta	accactcgtg	gcacccaact	gatcttcacg	atcttttact	ttcaccacgg	6480
ttttctgggtg	agcaaaaaaca	ggaaggcaca	atgcgcgcaa	aaaggggaata	agggcgacac	6540
ggaaaatgtg	aaatactcata	ctcttctctt	gtcaaatatta	ttgaagcatt	tacagggtt	6600
attgtctcat	gagcggtacg	atatttgaag	gtatttagaa	aaataaacaa	ataggggttc	6660
cgcgcacatt	tcgccaaaaa	gtgccacctg	acgtc			6695

<210> 16

<211> 6801

<212> DNA

<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence: Construct  
LBDSVP16

<400> 16

gacggatcgg	gagatctccc	gatccccctat	ggtcgactct	cagttacaatc	tgctctgatg	60
ccgcatagtg	aagccagtagt	ctgctccctg	ctgtgtgtgtt	ggaggtcgctg	gagtagtgcg	120
agcgcaaaat	ttaaagctaca	acaaggcgaag	gcttgaccga	caattgcatcg	aagaaactcgc	180
ttaggggttag	gcggttttgcg	ctgctttcgcg	atgtacgggc	cagatatacatg	cgttgacatt	240
gattattgac	tagttatttaa	tagtaaatcaa	ttacgggggtc	attagttcat	agcccatata	300
tggaagtccg	cgttacataaa	cttacggttaa	atggcccgcc	tggttgacgc	cccaacgacc	360
ccccccatt	gacgtcaata	atgacgtatg	ttcccatagt	aacgcgaata	gggactttcc	420
attgacgtca	atgggtggac	tattttacggt	aaactgccca	cttggcagta	catcaaagtg	480
atcatatgcc	aagtacgccc	cctattgacg	tcaatgacgg	taaatggccc	gctcggcagt	540
atgcccgtag	atcttccagat	tggaactttc	ctacttgcca	gtacatctac	gtattagcca	600
tcgctattac	catggtgatg	cgggtttggc	agttacatcaa	tgggcgtaga	tagcggtttg	660
actcacgggg	attttccaa	ctccacccca	ttgacgtcaa	tgggagtttg	ttttggcacc	720
aaaaatcaacg	ggactttcca	aaatgtcgta	acaactccgc	cccatgtacg	caaatggggc	780
gttagcgtgtg	acgggtggag	gtctatataa	gcagagactct	ctggctaaact	agagaaccca	840
ctgcttaactg	gcttattcgaa	attaatacga	ctcactatag	ggagacccaa	gctcggctagc	900
gttttaaacctt	aagcttttgta	ccgagctcgg	ccactactagt	ccagtggtggt	ggaattctcgt	960
cagcccggggg	gatctatggc	ccaggcgccg	ctcgagccct	atgcttgccc	tgctcaggtcc	1020
tcgcgatcgcc	gctttctctaa	ctcgggtcgt	ctgaagcgcc	atatccgcat	ccacacagcg	1080
cagaagcctt	tcctgtgtcgc	aatatgcatg	cgtaactctga	gtcgtagtga	ccctcttacc	1140
accacatccc	gcacccaac	agggcggaag	ccttttgctc	gtgcattttg	tcggagggaa	1200
tttgccagtg	gtgactgaacg	caagaggcat	accaaaatcc	atttaagaca	gagggactct	1260
agaactagtg	accgaagag	agggagaatg	ttgaacacaa	agccgcagag	agatgtagtg	1320
gagggccagg	gtgaagtggg	gtctgctgga	gacatgagag	ctgcacaaact	ttggccaagc	1380
ccgctcatca	tcaaacgctc	taagaagaac	agcctggcct	tgctccctgac	ggccgaccag	1440
atggtcagtg	ccttgtttgca	tgctgagccc	cccataactct	attccagata	tgatctctacc	1500
agacccttca	gtgaagcttc	gatgatgggc	ttactgacca	acctggcgca	cagggaagctg	1560
gtttcatcatg	tcaactgggc	gaagagggtg	ccaggctttg	tggaattgac	ccctcatgat	1620
cagggtccacc	ttctagaatt	tgcttggtta	gagatccctga	ttgtttggtct	cgcttcggcg	1680
ttccattggagc	accacgggaa	gctactgttt	gctcctaact	tgctcttgga	cagggaaccaa	1740
ggaaaaattgt	tagagggcat	ggtggagatc	ttcgacatgc	tgctggtctac	atcatctcgg	1800
ttccgatga	tgaactctga	gggagaggag	ctttgtgtcc	tcaaactctat	tattttgtct	1860
aatctctggag	tgtaacacat	ctctgccagc	accctgaagt	ctctggaaga	gaaggaccat	1920
atccacggctg	tcctggacaa	gatcacagac	actttgatcc	acctgatgca	caggcgaggc	1980
ctgaccctgc	agcagcgaca	ccagcggctg	gcccgactcc	tctctatcct	ctcccacatc	2040
aggcacatga	gtaaacaaag	catggagcat	ctgtacagca	tgaagtgcga	gaactgtggtg	2100

[illegible]

acaggcatgc	tgggtgcacg	ctcgtcgctt	ggatggtgct	cattcagctc	cggttcccaa	6180
cgatcaaggc	gagttacatg	atcccccatg	ttgtgcaaaa	aagcggttag	ctccttcggt	6240
ctccgatcgc	ttgtcagaag	taagttggcc	gcagtggtat	cactcatggt	taaggcagca	6300
ctgcaataat	ctcttactgt	catgccatcc	gttaagatgct	ttctgtgtgac	tggtgagtag	6360
tcaaccaagt	cattctgaga	atagtgtatg	cgccgaccga	gttgctctctg	ccggcgctca	6420
ataccgggga	ataccgggcc	acatagcaga	actttaaaaa	ggtccatcat	tggaaaaagt	6480
ttctcggggc	gaaaaactct	aaggatctta	ccgctgttga	ccagcgtttc	gatgttaacc	6540
actcgtgcac	ccaactgatc	tttactttca	ccagcgtttc	ccagcgtttc	tggtgagcga	6600
aaaaacaggaa	ggcaaaatgc	cgcaaaaaag	ggaataaggg	cgacacggaa	atgttgtaata	6660
ctcactactct	ctctttttca	atattattga	agcattttac	aggggtattg	tctcatgagc	6720
ggatcacat	ttgaatgtat	ttagaaaaat	aaacaaatag	gggttccgcg	cacatttccc	6780
cgaaaaagtc	cacctgacgt	c				6801

<210> 17

<211> 1551

<212> DNA

<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence: Construct  
VP16C7ER

<400> 17

gctagcgcca	ccatggggcg	cgccggcgct	ccccgaccg	atgtcagcct	gggggacgag	60
ctccacttag	acggcgagga	cgtggcgatg	gcgcgatgcc	acgcgctaga	cgatttcgat	120
ctggacatgt	tgggggacgg	ggattccccc	gggtccggat	ttaccoccca	cgactccgcc	180
ccctacggcg	ctctgggat	ggccgacttc	gagtttgagc	agatgtttac	cgatgccttt	240
ggaatttgac	agtagcggtt	aattaacaag	cttggggccc	agggcgccct	cgagccctat	300
gcttgccctg	tcgagtctgt	cgatcgccgc	ttttctaaat	cggtgatctc	gaagcgccat	360
atccgcattc	acacaggcca	gaagcccttc	cagtgctgaa	tatgcatgct	taacttcagt	420
cgtagtgaac	accttaccac	ccacatccgc	accacacacg	gcgagaagcc	ttttgcctgt	480
gacattttgt	ggagagaagt	tgccaggagt	gatgaacgca	agaggcatat	caaaatccat	540
ttgaagacga	aggactctag	aactagtggc	caggccggcc	agggggatcc	acgaaatgaa	600
atgggtgctt	caggagacgt	gagggctgcc	aacctttggc	caagccctct	tgtgattaa	660
cacactaaga	agaatagccc	tgccctgttc	ttgacagctg	accagatggt	cagtgccctg	720
ttggatgctg	aaccgcggcc	gatctatttc	gaatatgato	cttctagact	cttcagttaa	780
gcctcaatga	tgggcttatt	gaccaacctc	gcagataggg	agcttggtta	tatgatcaac	840
tgggcaaga	gagtgccagg	ctttggggac	ttgaattctc	atgatcaggt	ccacctttct	900
gagtggtgct	ggctggagat	cttgatgatt	gggtctgctc	ggcgctccat	ggaacaccgc	960
gggaagctcc	tgtttgcctc	taacttgctc	ctggacagga	atcaaggtaa	atgtgtggaa	1020
ggcatggtgg	agatctttga	catgttgctt	gctacgtcaa	gtcgggtccg	catgatgaac	1080
ctgcagggtg	aagagtttgt	gtgcctcaaa	ttcatcattt	tgcttaattc	cggagtgtag	1140
acgtttctgt	ccagcacctt	gaagtctctg	gaagagaagg	accacatcca	ccgtgtcctg	1200
gacaagatga	gacagacttt	gattccacct	atggccaaag	ctgcctctgac	cttcgacgag	1260
cagcatcgcc	gcctagctca	gctcctcttc	attctttccc	atatccgcca	catgatgaac	1320
aaaggcatgg	agctactcta	caacatgaaa	tgcaagaacg	ttgtgcccct	ctatgacctg	1380
ctctgggaga	tgttgagtag	ccacgccttt	catgccccag	cgactgcgat	ggagatgcct	1440
ccagagggag	ccagccagac	ccagctggcc	accaccagct	ccacttcagc	aatttcttta	1500
caaacctact	acataccccc	ggaagcagag	ggcttcccca	acacgatctg	a	1551

<210> 18

<211> 1404

<212> DNA

<213> Artificial Sequence

<220>

<220>

<223> Description of Artificial Sequence: Construct  
VP16C7PR

<400> 18

gctagcgcca	ccatggggcg	cgccggcgct	ccccgaccg	atgtcagcct	gggggacgag	60
ctccacttag	acggcgagga	cgtggcgatg	gcgcgatgcc	acgcgctaga	cgatttcgat	120
ctggacatgt	tgggggacgg	ggattccccc	gggtccggat	ttaccoccca	cgactccgcc	180



<400> 21  
cgccgcccgc gccgcgcgcg cgtgggcg

28

<210> 22  
<211> 35  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 22  
Met Lys Leu Leu Glu Pro Tyr Ala Cys Pro Val Glu Ser Cys Asp Arg  
1 5 10 15  
Arg Phe Ser Lys Ser Ala Asp Leu Lys Arg His Ile Arg His Thr Gly  
20 25 30  
Glu Lys Pro  
35

<210> 23  
<211> 29  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 23  
Tyr Ala Cys Pro Val Glu Ser Cys Asp Arg Arg Phe Ser Lys Ser Ala  
1 5 10 15  
Asp Leu Lys His Ile Arg Ile His Thr Gly Glu Lys Pro  
20 25

<210> 24  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 24  
cctcgccgcc gcgggttttc ccgcgcccc gagg

34

<210> 25  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule  
<220>

<221> nnn= a mixture of all 64 existing triplets and its complement  
<222> 26-28 and 7-9 respectively

```

<400> 25
ggacgcgnnnc gcgggttttc ccgcggnnngc gtcc 34

<210> 26
<211> 66
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
molecule

<400> 26
gcgagcaagg tcgcggcagt cactaaaaaga ttgcccgcac tctgggcatt tatacggttt 60
ttcacc 66

<210> 27
<211> 74
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
molecule

<400> 27
gtgactgcgc cgaccttgct cgccatcaac gcactcatatc tggcgagaag ccatacaaat 60
gtccagaatg tggc 74

<210> 28
<211> 81
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
molecule

<400> 28
ggtaagtctt tctctcagag ctctcacctg gtgcgccacc agcgtaccca cacgggtgaa 60
aaaccgtata aatgccaga g 81

<210> 29
<211> 58
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
molecule

<400> 29
acgcaccagc ttgtcagagc ggctgaaaaga cttgccacat tctggacatt tgtatggc 58

<210> 30
<211> 87
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
molecule

<400> 30
gaggaggagg aggtggccca ggcggccctc gagccggggg agaagcccta tgcttgtccg 60
gaatgtggta agtccttc tcagagc 87

```

<210> 31  
 <211> 81  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Recombinant molecule  
  
 <400> 31  
 gaggaggagg agctggccgg cctggccact agttttttta ccggtgtgag tacgttggtg 60  
 acgcaccagc ttgtcagagc g 81  
  
 <210> 32  
 <211> 44  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Recombinant molecule  
  
 <400> 32  
 gaggaggagg ctagcgggat gtggtcttgc cctcaacagg tagg 44  
  
 <210> 33  
 <211> 41  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Recombinant molecule  
  
 <400> 33  
 gaggaggaga agcttctcgt ccgcctcccg cggcgctccg c 41  
  
 <210> 34  
 <211> 48  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Recombinant molecule  
  
 <400> 34  
 gaggaggagg ctagccgatg tgactgtctc ctcccaaatt tgtagacc 48  
  
 <210> 35  
 <211> 42  
 <212> DNA  
 <213> Artificial Sequence  
  
 <220>  
 <223> Description of Artificial Sequence: Recombinant molecule  
  
 <400> 35  
 gaggaggaga agcttggtgc tcactgcggc tccggcccca tg 42  
  
 <210> 36  
 <211> 11  
 <212> PRT  
 <213> Artificial Sequence  
  
 <220>



<210> 42  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 42  
ccacttaaat gtgaaagtcg tacgccggcc 30

<210> 43  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 43  
tatggggggc tcagcatcca acaaggcact 30

<210> 44  
<211> 48  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 44  
cctactacta gtgaccgaag aggagggaga atgttgaaac acaagcgc 48

<210> 45  
<211> 42  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 45  
cctactacta gtagtattca aggacataac gactatatgt gt 42

<210> 46  
<211> 39  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 46  
tatcatgtgc ggccgcttac ttagttaccc cggcagcat 39

<210> 47  
<211> 39  
<212> PRT  
<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Recombinant molecule

<400> 47

Pro Ala Asp Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Pro Ala Asp  
1 5 10 15

Ala Leu Asp Asp Phe Asp Leu Asp Met Leu Pro Ala Asp Ala Leu Asp  
20 25 30

Asp Phe Asp Leu Asp Met Leu  
35

<210> 48

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Recombinant molecule

<400> 48

gatccaaagt cgcgtgggcg cagcgccac gcgatcaaag a 41

<210> 49

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Recombinant molecule

<400> 49

gatccaaagt ccaggcgagc gcgtgggcyg cagatcaaag a 41

<210> 50

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Recombinant molecule

<400> 50

gatccaaagt cgcgtgggcyg caggcgcyag cgtgggcyga tcaaaga 47

<210> 51

<211> 41

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: Recombinant molecule

<400> 51

gatccaaagt cgcgtgggcyg cagcgccac gcgatcaaag a 41

<210> 52

<211> 41

<212> DNA

<213> Artificial Sequence

<220>		
<223>	Description of Artificial Sequence: Recombinant molecule	
<400>	52 gatccaaagt cgcgtagggcg cactccggcc ccgatcaaag a	41
<210>	53	
<211>	41	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	Description of Artificial Sequence: Recombinant molecule	
<400>	53 gatccaaagt cggggccgga gactccggcc ccgatcaaag a	41
<210>	54	
<211>	23	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	Description of Artificial Sequence: Recombinant molecule	
<400>	54 gccggagcca tggggccgga gcc	23
<210>	55	
<211>	21	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	Description of Artificial Sequence: Recombinant molecule	
<400>	55 cgctccctct caggcgagg g	21
<210>	56	
<211>	21	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	Description of Artificial Sequence: Recombinant molecule	
<400>	56 ggcgccact gtggggcggg c	21
<210>	57	
<211>	41	
<212>	DNA	
<213>	Artificial Sequence	
<220>		
<223>	Description of Artificial Sequence: Recombinant molecule	
<400>	57 gaggaggagg gccggccggg aagcgtgca ggaagagcgg c	41

<210> 58  
<211> 43  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant molecule

<400> 58  
gaggaggagg gcgcgcccag tcatttggtg cggcgctcc agc 43

<210> 59  
<211> 42  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant molecule

<400> 59  
gaggaggagt taattaaagt catttggtgc ggcgcctcca gc 42

<210> 60  
<211> 47  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant molecule

<400> 60  
gaggaggagg gccggccggg gtggcgccca agactttgtt aagaagg 47

<210> 61  
<211> 50  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant molecule

<400> 61  
gaggaggagg gccagggcgg ccggtggcgg ccaagacttt gttaagaagg 50

<210> 62  
<211> 45  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant molecule

<400> 62  
gaggaggagg gcgcgcccgg catgaacgtc ccagatctcc togag 45

<210> 63  
<211> 46  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant

[illegible]

-40 of 46-



Ser Gly Arg Leu Leu Glu Gln Lys Ala Ala Ser Cys His Asn Ser Tyr  
180 185 190

atc gtc ctg tgc att gag aat agc ttc atg acc tct ttc tcc aaa tag 624  
Ile Val Leu Cys Ile Glu Asn Ser Phe Met Thr Ser Phe Ser Lys \*  
195 200 205

<210> 71  
<211> 207  
<212> PRT  
<213> Muridae

<400> 71  
Met Glu Thr Asp Thr Leu Leu Leu Trp Val Leu Leu Leu Trp Val Pro  
1 5 10 15  
Gly Ser Thr Gly Asp Ala Ala His Thr His Gln Asp Phe Gln Pro Val  
20 25 30  
Leu His Leu Val Ala Leu Asn Thr Pro Leu Ser Gly Gly Met Arg Gly  
35 40 45  
Ile Arg Gly Ala Asp Phe Gln Cys Phe Gln Gln Ala Arg Ala Val Gly  
50 55 60  
Leu Ser Gly Thr Phe Arg Ala Phe Leu Ser Ser Arg Leu Gln Asp Leu  
65 70 75  
Tyr Ser Ile Val Arg Arg Ala Asp Arg Gly Ser Val Pro Ile Val Asn  
85 90 95  
Leu Lys Asp Glu Val Leu Ser Pro Ser Trp Asp Ser Leu Phe Ser Gly  
100 105 110  
Ser Gln Gly Gln Val Gln Pro Gly Ala Arg Ile Phe Ser Phe Asp Gly  
115 120 125  
Arg Asp Val Leu Arg His Pro Ala Trp Pro Gln Lys Ser Val Trp His  
130 135 140  
Gly Ser Asp Pro Ser Gly Arg Arg Leu Met Glu Ser Tyr Cys Glu Thr  
145 150 155 160  
Trp Arg Thr Glu Thr Thr Gly Ala Thr Gly Gln Ala Ser Ser Leu Leu  
165 170 175  
Ser Gly Arg Leu Leu Glu Gln Lys Ala Ala Ser Cys His Asn Ser Tyr  
180 185 190  
Ile Val Leu Cys Ile Glu Asn Ser Phe Met Thr Ser Phe Ser Lys  
195 200 205

<210> 72  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Integrin  $\beta 3$  (B3B) target sequence

<400> 72  
gcctgagagg gagcggtg 18

<210> 73  
<211> 18  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Integrin  $\beta 3$  (B3C) target sequence

<400> 73  
ggaggggacg cgggtgggt 18

<210> 74  
<211> 20  
<212> DNA  
<213> Artificial Sequence

```

<220>
<223> Description of Artificial Sequence: ErbB-2 (E2B2) target sequence

<400> 74
gtgtgagaac ggctgcaggc                                20

<210> 75
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ErbB-2 (E2C) target sequence

<400> 75
ggggccggag ccgcagtg                                    18

<210> 76
<211> 18
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: ErbB-2 (E2D) target sequence

<400> 76
gcagttggag ggggcgag                                    18

<210> 77
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
      molecule

<400> 77
Gln Ser Ser Asn Leu Val Arg
 1                      5

<210> 78
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
      molecule

<400> 78
Asp Pro Gly Asn Leu Val Arg
 1                      5

<210> 79
<211> 7
<212> PRT
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: Recombinant
      molecule

<400> 79
Arg Ser Asp Asn Leu Val Arg

```

1

5

<210> 80  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 80  
Thr Ser Gly Asn Leu Val Arg  
1 5

<210> 81  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 81  
Gln Ser Gly Asp Leu Arg Arg  
1 5

<210> 82  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 82  
Asp Cys Arg Asp Leu Ala Arg  
1 5

<210> 83  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 83  
Arg Ser Asp Asp Leu Val Lys  
1 5

<210> 84  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 84  
Thr Ser Gly Glu Leu Val Arg  
1 5

<210> 85  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 85  
Gln Arg Ala His Leu Glu Arg  
1 5

<210> 86  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 86  
Asp Pro Gly His Leu Val Arg  
1 5

<210> 87  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 87  
Arg Ser Asp Lys Leu Val Arg  
1 5

<210> 88  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 88  
Thr Ser Gly His Leu Val Arg  
1 5

<210> 89  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 89  
Gln Ser Ser Ser Leu Val Arg  
1 5

<210> 90  
<211> 7

<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 90  
Asp Pro Gly Ala Leu Val Arg  
1 5

<210> 91  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 91  
Arg Ser Asp Glu Leu Val Arg  
1 5

<210> 92  
<211> 7  
<212> PRT  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: Recombinant  
molecule

<400> 92  
Thr Ser Gly Ser Leu Val Arg  
1 5